# Intrinsic labelling of different foods with stable isotope of zinc ($^{67}$Zn) for use in bioavailability studies

BY THOMAS E. FOX*, SUSAN J. FAIRWEATHER-TAIT, JOHN EAGLES AND S. GABRIELLE WHARF

*AFRC Institute of Food Research, Norwich Laboratory, Colney Lane, Norwich NR4 7UA*

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**Intrinsically-labelled foods are required to validate extrinsic-labelling techniques used to study the bioavailability of trace elements. Wheat (*Triticum aestivum*), peas (*Pisum sativum*), goat's milk, human milk, eggs and chicken meat were selected for intrinsic-labelling studies with $^{67}$Zn. Peas were grown hydroponically in enriched nutrient solution and wheat was grown in sand and watered with enriched nutrient solution. Some of the wheat plants were also given stem injections of $^{67}$Zn solution. Eggs and chicken meat were prepared by administering $^{67}$Zn intravenously to chickens, and human milk was collected after an oral dose of $^{67}$Zn in a cola drink. All the foods investigated were sufficiently enriched with $^{67}$Zn for Zn absorption studies except wheat prepared by the sand and water-culture method.**

**Intrinsically-labelled foods: Bioavailability: Zn**

Zinc is a trace element of particular interest because of recent reports of marginal or inadequate intake among certain groups of the population (Hambidge, 1989). Like many other inorganic nutrients, Zn is not particularly well absorbed from the diet, and an understanding of factors affecting its bioavailability is, therefore, required.

Until recently, much of the work on the bioavailability of Zn has involved the use of radioisotopes as an extrinsic label in various test meals. To date, only a few studies have been carried out to validate the extrinsic tag (Evans & Johnson, 1977; Janghorbani *et al.* 1982; Meyer *et al.* 1983; Flanagan *et al.* 1985; Gallaher *et al.* 1988), and the assumption that it behaves in the same way and that its absorption is the same as endogenous forms of Zn is questionable. Absorption studies involving human subjects raise the ethical question of the use of radioisotopes, but the comparatively recent introduction of the use of stable isotopes in nutrition research offers a safer alternative, both ethically and in the preparation of intrinsically-biosynthetically-labelled foods, where the short half-life of many radioisotopes is a limiting factor. The advantages of intrinsic labelling are that the mineral is deposited in the same manner and associated with the same constituents as the endogenous Zn that occurs naturally within the food.

When studying the absorption of a mineral, true or apparent, from an intrinsically-labelled test meal, the tracer isotope must be present in sufficient concentrations to be measurable in the faeces after excretion and faecal dilution. In the present study we investigated the feasibility of preparing $^{67}$Zn intrinsically-labelled peas (*Pisum sativum*), wheat (*Triticum aestivum*), chicken meat, eggs, goat's and human milk sufficiently enriched for use in metabolic studies.

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Table 1. *Nutrient solution for peas* (Pisum sativum) *and wheat* (Triticum aestivum)

| Compound | Concentration (mg/l) |
|---|---|
| $NH_4H_2PO_4$ | 230·0 (high) |
| $NH_4H_2PO_4$ | 115·0 (low) |
| $KNO_3$ | 606·0 |
| $Ca(NO_3)_2.4H_2O$ | 944·0 |
| $MgSO_4.7H_2O$ | 492·0 |
| $H_3BO_3$ | 2·86 |
| $MnCl_2.4H_2O$ | 1·81 |
| $CuSO_4.5H_2O$ | 0·08 |
| $H_2MoO_4.H_2O$ | 0·09 |
| Fe EDTA | 5·0 |
| $^{67}ZnCl_2$ | 0·05 |

## MATERIALS AND METHODS

### *Isotope*

For all the foods, the isotope used for intrinsic labelling was elemental Zn, 91·9 atom% $^{67}Zn$ (Technical and Optical Equipment, Edgware Road, London).

### *Peas*

Forty-eight dwarf pea seeds (var. Chemin Long) obtained from John Innes Institute, Norwich (JI 296), were sown at a depth of 50 mm in washed silica sand (double arches pit no. 21; Joseph Arnolds and Sons Ltd, Easton Way, Heaton Reach, Leighton Buzzard, Bedfordshire) in plastic buckets (six per bucket). The seeds were watered with a modified Hoagland and Arnon nutrient solution (Weaver, 1985) which has a high phosphate content favoured by leguminous plants (Table 1). At 3 weeks the plants were transferred to plastic buckets with 10 mm holes drilled in the lids. The buckets were filled to the top with the modified nutrient solution and the plants held so that the stem protruded through the lids with the root system in the nutrient solution. The buckets were blacked out to reduce light penetration and taped with white tape on the outside to prevent temperature increase. The plants were kept in a well-ventilated, low-temperature greenhouse.

The nutrient solution was changed every 10 d and aerated continuously with compressed air via plastic tubes. The pH was monitored throughout and kept between 5·5 and 6·4 (Weaver, 1985) with the addition of nitric acid. At 8 weeks after sowing the phosphate level in the nutrient solution was reduced to 31 mg/l on flowering (Layrisse *et al.* 1969) and the isotope $^{67}Zn$ introduced into the nutrient solution in place of zinc sulphate as 56·5 $\mu$l/l nutrient solution. At 12 weeks after sowing the peas were harvested and dried in an oven at 80°, ground to a flour; total weight 127 g.

### *Wheat*

Approximately 200 self-pollinating winter wheat seeds were planted in acid-washed silica sand in 20 litre black plastic buckets which had been whitened on the outside. The sand was obtained from Arnold Quarries double arches pit no. 21 as washed silica sand. It was soaked in concentrated hydrochloric acid (180 ml/l; BDH Chemicals Ltd, Poole, Dorset) for 1 week before leaching with deionized water and then nutrient solution until the effluent pH was the same as that added, and there was no change in pH after being left for 24 h in contact with the sand.

The wheat seeds were sown in November at a depth of 25 mm, twelve in each bucket and watered with a modified Hoagland and Arnon nutrient solution (Weaver, 1985) and kept in a cool, well-ventilated greenhouse. Just before anthesis, the $ZnSO_4$ was replaced with $^{67}ZnCl_2$ at a concentration of 56 $\mu$l/l nutrient solution. On initiation of flowering, half the wheat was stem-injected with $^{67}Zn$ solution prepared by dissolving $^{67}Zn$ in concentrated Aristar HCl (BDH Chemicals Ltd) and adding 0·1 M-citrate and 0·1 M-sodium citrate. The pH was adjusted to 5·5 with sodium bicarbonate. The final concentration of Zn in the solution was 0·158 mg/ml. This solution was injected just below the base of the emergence spike using a 16 mm 25 gauge sterile hypodermic needle, taking care to insert the needle into the stem only as far as the cavity of the pith (Weaver, 1985). A second needle was used as a vent hole just above the first joint to reduce turgor pressure whilst injecting approximately 0·2 ml. The rest of the wheat was watered with the $^{67}Zn$-enriched nutrient solution. The wheat was harvested in July the following year and dried in an oven at 80° for 48 h. The seeds were separated from the chaff by hand and then milled through a 250 $\mu$m gauge sieve. The total yield of wheat flour was 560 g.

*Goat's milk*

Enriched goat's milk was prepared by intravenously injecting a Cameroon pygmy goat in the jugular vein with a single dose of 10·2 ml of a $^{67}Zn$ solution. The solution was made up by dissolving 9·725 mg in 10 ml Aristar concentrated HCl and adding 1 mmol tri-sodium citrate with distilled water to dissolve. The pH was adjusted to 7·36 by the addition of sodium bicarbonate to give a final volume of 12 ml with a Zn concentration of 0·8104 mg/ml. The goat was milked for 5 d, morning and afternoon, yielding approximately 1·5 pints per milking. Each batch of milk was freeze-dried after collection.

*Chicken meat*

Ten Ross I, broiler-type cockerels, weighing 2·025 kg each, obtained from a commercial hatchery, were fed on growers pellets (Allen and Page Ltd, Norwich) *ad lib.* with water for 27 d. The chicken meat was labelled with $^{67}Zn$ by injecting 1 ml of a solution (1·84 mg/ml) prepared as described previously into the brachial vein on alternate wings each day for 5 d, starting on the 23rd day. The chickens were killed after 1 week, bled, defeathered and eviscerated. The breast and leg meat were taken immediately afterwards and cooked in a microwave at 400 W for 10 min. Each bird yielded approximately 420 g meat. The cooked meat was minced in a Magimix 2800S food blender and freeze-dried.

*Eggs*

Enriched eggs were collected from a hen injected with 3 ml of a solution, prepared by dissolving 0·1053 g $^{67}Zn$ in 1·25 ml concentrated HCl and made up to 100 ml with distilled water, whilst adding sodium hydroxide to adjust the pH to 6·25. A further injection of 3 ml was given the following day, after the hen had laid one egg. The eggs were collected daily, cooked individually as scrambled egg in a microwave and freeze-dried.

*Human milk*

Enriched human milk was obtained by orally administering 30 ml Zn solution (containing 24·4 mg $^{67}Zn$) in 330 ml cola drink at 10.00 hours, after an overnight fast, to a lactating woman at 3·5 months post partum. No food was consumed until 13.00 hours. Milk was expressed every 4 h, starting at 14.00 hours until 14.00 hours the following day. All milk samples were combined and freeze-dried. The study was approved by the Institute of Food Research Ethical Committee.

Table 2. *Total zinc concentration, $^{64}Zn$:$^{67}Zn$ ratio, and $^{67}Zn$ concentration of foods intrinsically labelled with $^{67}Zn$**

| Food | Total Zn (μg/g dry wt) | $^{64}Zn$:$^{67}Zn$ ratio (natural 11·81) | | $^{67}Zn$ concentration (μg/g dry wt) |
|---|---|---|---|---|
| | | Mean | % RSD | |
| Peas (*Pisum sativum*) | 46·5 | 2·62 | 0·6 | 6·5 |
| Wheat (*Triticum aestivum*) | | | | |
| Injected | 54·1 | 1·57 | 0·9 | 12·6 |
| Non-injected | 18·2 | 10·11 | 0·4 | 0·1 |
| Human milk | 7·1 | 6·87 | 0·4 | 0·2 |
| Chicken | 45·7 | 3·05 | 0·1 | 5·4 |
| Egg | 30·4 | 2·17 | 0·5 | 5·2 |

RSD, residual standard deviation.
* For details of procedures, see pp. 58–59.

### *Atomic absorption spectroscopy (AAS)*

For each sample analysed, a portion of dried food was weighed into a silica crucible, ashed at 480° for 48 h, and the ash mixed thoroughly. Subsamples of the ash were analysed for Zn by dissolving 0·05–0·1 g ash in 2 ml hot 11·8 M-Aristar HCl (BDH Chemicals Ltd) and making the solution up to 20 ml with distilled water. The Zn concentration was measured on a Pye Unicam PU900 AAS (Philips, Cambridge) and readings checked against NBS reference materials, wheat or liver (Office of Standard Reference Materials, Washington DC, USA).

### *Mass spectrometry*

Enrichment with $^{67}Zn$ was determined using 2 g samples of the ash that had been refluxed with 2 ml 11·8 M-Aristar HCl for 24 h, then dried and taken up in 2 ml of the acid and diluted with quartz-distilled water to 10 ml. The solution was passed through an AG 1-X8 anion exchange resin, 200–400 mesh, chloride form (Bio Rad Laboratories, California, USA), previously swelled for 48 h in quartz-distilled water and collected as 1·5 ml fractions and dried under a 1 KW lamp in a laminar flow cabinet. The dried fractions were analysed using a thermal ionization mass spectrometer (Finnigan Mat., Bremen, W. Germany). The rhenium filaments were prepared by adding 10 μl dilute silica gel to the filament and drying. The fractions were dissolved in 12 μl 12 M-Aristar nitric acid and 5 μl together with 1 μl orthophosphoric acid, added to the filament. The sample $^{64}Zn$:$^{67}Zn$ ratio was determined along with standard Zn sample and blank (Eagles *et al.* 1989).

## RESULTS AND DISCUSSION

All the food samples prepared were sufficiently enriched with $^{67}Zn$ for bioavailability studies measuring apparent Zn absorption, with the exception of the non-stem-injected wheat. The foods with their total Zn and $^{64}Zn$:$^{67}Zn$ ratios are shown in Table 2. The concentration of Zn in peas grown hydroponically, enriched with $^{67}Zn$, was similar to that of control peas grown in silica sand, watered with the same nutrient solution without $^{67}Zn$. However, the Zn concentrations were higher than those of 35 mg/g dry weight given in *McCance and Widdowson's The Composition of Foods* (Paul & Southgate, 1978).

Wheat showed a marked difference between the stem-injected and non-stem-injected wheat, the former having a much higher total Zn and $^{67}Zn$ concentration. The difference

Table 3. *Enrichment and zinc concentration of dried goat's milk after a single intravenous dose of $^{67}Zn$**

| Time interval after dose (h) | Zn concentration ($\mu$g/g) | $^{64}Zn$:$^{67}Zn$ ratio | |
|---|---|---|---|
| | | Mean | % RSD |
| 0 | 33·9 | 11·78 | 0·4 |
| 4 | 39·9 | 3·21 | 0·2 |
| 16 | 40·4 | 3·81 | 0·1 |
| 42 | 40·7 | 4·33 | 0·2 |
| 48 | 40·6 | 4·93 | 0·2 |
| 66 | 48·6 | 7·28 | 0·1 |

RSD, residual standard deviation.
* For details of procedures, see p. 59.

in total Zn was almost entirely due to the amount of $^{67}Zn$ incorporated into the grain. The total concentration of Zn was within the range reported by Burk & Solomons (1985) of 21–63 $\mu$g/g and close to the average value of 47 $\mu$g/g reported by Davis *et al.* (1984). Similar values were reported by Starks & Johnson (1985) for stem-injected wheat of 59·9 $\mu$g/g when injecting $^{65}Zn$ and $ZnSO_4$ together. However, this group found that with hydroponics, a Zn concentration of 20·1 $\mu$g/g was obtained, which was less than half the concentration of the wheat we prepared with the sand and water method. The low enrichment associated with hydroponics could mean that the introduction of the isotope on anthesis is not the optimal time for isotope application. From anthesis up to seed formation, vegetative growth of the wheat has been found to stop (Noggle & Fritz, 1976), and during this period, the minerals deposited in the leaves are translocated to the reproductive areas of the plant (Noggle & Fritz, 1976; Waldren & Flowerday, 1979). It would seem likely therefore, that the Zn assimilated during growth of the plant would be the bulk of the Zn making up seed formation. On this basis it would seem probable that a higher incorporation of isotopes into the seed would be achieved if the application of the isotope was made during the latter half of vegetative growth and not at the onset of anthesis.

The chicken meat was found to be sufficiently enriched for absorption studies with a $^{64}Zn$:$^{67}Zn$ ratio nearly four times that found naturally. The total Zn concentration was higher than values of 20 $\mu$g/g published in food composition tables (Paul & Southgate, 1978), and those found by Janghorbani *et al.* (1982), (approximately 17 $\mu$g/g) who fed cockerel chickens $^{68}Zn$ mixed with the feed.

The $^{64}Zn$:$^{67}Zn$ ratio of dried goat's milk and Zn concentrations with time-interval after dose administration are shown in Table 3. Milk collect 4 h after intravenous administration had the highest enrichment, with a $^{64}Zn$:$^{67}Zn$ ratio 3·7 times the natural ratio. Enrichment of $^{67}Zn$ in the milk gradually declined over the following 66 h to a $^{64}Zn$:$^{67}Zn$ ratio of 7·28. Total milk Zn concentration increased from 34 to 48 $\mu$g/g. Similar results were observed by Serfass *et al.* (1987) who found that peak enrichment of cows' milk was 4–8 h after a single dose of 26·8 mg $^{67}Zn$, with an increase in concentration following the administration of isotope.

Enriched human milk samples had $^{64}Zn$:$^{67}Zn$ ratios which were nearly twice that of natural abundance. There was a significant difference in the human-milk Zn concentrations between non-enriched and enriched samples, with the enriched samples being 34% lower than the non-enriched samples. A period of 2 weeks had elapsed between samples taken

Table 4. *Enrichment of eggs after two consecutive intravenous doses of $^{67}Zn$ given to a hen**

| Period after dose (d) | $^{64}Zn$:$^{67}Zn$ ratio | |
|---|---|---|
| | Mean | % RSD |
| 0 | 11·80 | 0·7 |
| 1 | 11·82 | 0·7 |
| 2 | 11·51 | 0·7 |
| 3 | 5·55 | 0·3 |
| 4 | 2·19 | 0·3 |
| 5 | 1·56 | 0·2 |
| 6 | 1·14 | 0·4 |
| 7 | 1·16 | 0·1 |
| 10 | 1·23 | 0·2 |
| 14 | 1·73 | 0·3 |

RSD, residual standard deviation.
* For details of procedures, see p. 59.

from non-enriched milk collected and the enriched samples taken after administration of the isotope. Krebs *et al.* (1985) observed that human milk Zn concentrations fell between the first and ninth month of lactation from 2·65 (SE 0·81) to 0·67 (SE 0·40) $\mu$g/ml, with the most rapid decline being between the first and third month. It seems likely, therefore, that the lower level of Zn found in the enriched samples was due to the fall in concentration of Zn at the end of the third month of lactation rather than isotope administration.

Egg samples collected over 16 d after $^{67}Zn$ administration, analysed for $^{67}Zn$ and total Zn, are shown in Table 4. Peak incorporation of $^{67}Zn$ was 6 d post injection. Meyer *et al.* (1983) obtained freeze-dried eggs with Zn levels of 108·25 $\mu$g/g after feeding chickens a diet containing $^{65}ZnCl_2$.

In all the samples of food, the Zn concentrations in the enriched foods were within the normal range present in non-enriched samples. The method of administration may affect the way the mineral is naturally bound in the food and this may limit the validity of an intrinsic label, and although there is no evidence to suggest this, investigations into the bound forms of the isotope compared with natural samples may need to be carried out. The mode of incorporation of $^{67}Zn$ into the food from a solution containing $^{67}ZnCl_2$ requires detailed examination in terms of its natural deposition and association with components within the food material.

It seems likely that foods which are formed in a relatively short time such as milk and eggs, deposit the Zn isotope in the same manner as natural Zn since these foods are synthesized almost entirely from constituents of the blood every 24 h or less. Foods formed over a much longer period of time such as wheat, peas and chicken meat may be more dependent on the time of dosing and chemical form of the isotope administered as to whether the isotope is deposited naturally, or stored in its administered or another form in cell vacuoles or intracellular spaces. Investigations into the form and distribution of isotopes in meat after intravenous administration are needed. There is some controversy over the fate of stem-injected Zn in plants. Weaver (1985) has reported that $^{65}Zn$ injected into maize and wheat stems was found to be distributed in a similar pattern to total Zn. However, a number of reports indicate that wheat does not contain a continuous xylem system into the kernels (Zee & O'Brien, 1970; Hamilton & O'Brien, 1979; Simpson *et al.* 1983) and that specific transfer cells exist that communicate with the phloem. Thus, it

would appear that substances injected into the stem of wheat may not accumulate in the kernels unchanged.

Although the chemical forms of $^{67}Zn$ in enriched foodstuffs have not yet been characterized, the present study has demonstrated that it is possible to prepare a range of foods sufficiently enriched for human bioavailability studies. This can be achieved at reasonable cost and without altering the Zn concentrations from those found naturally.

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