Iron bioavailability of cocoa powder as determined by the Hb regeneration efficiency method

Katsuhiko Yokoi[1,2]*, Aki Konomi[1] and Miki Otagi[2]

[1]*Department of Human Nutrition, Seitoku University Graduate School, 550 Iwase, Matsudo, Chiba 271-8555, Japan*
[2]*Department of Human Nutrition, Faculty of Humanities, Seitoku University, 550 Iwase, Matsudo, Chiba 271-8555, Japan*

Fe deficiency is a public-health problem worldwide, and effective measures for preventing Fe deficiency are needed. The aim of the present study was to determine the bioavailability of Fe in cocoa using the Hb regeneration efficiency (HRE) method. Thirty-five F344/N male weanling rats were fed a low-Fe diet for 4 weeks to deplete body Fe stores. Then, four groups of seven animals each were repleted for 20 d using a modified AIN-93G diet fortified with ferrous sulphate, ferric citrate or two brands of cocoa powder to provide a total dietary Fe concentration of 20 mg/kg. As a negative control, seven rats were maintained on the low-Fe diet. The HRE were 0·733, 0·350, 0·357 and 0·336 for ferrous sulphate, ferric citrate and the two brands of cocoa powder, respectively. The relative biological values (RBV), defined as the ratio of the sample HRE to that of ferrous sulphate, were 0·478, 0·488 and 0·459 for ferric citrate and the two brands of cocoa powder, respectively. The Fe bioavailability of cocoa was significantly less than that of ferrous sulphate and was similar to that of ferric citrate. The difference in Fe bioavailability between the two brands of cocoa powder was negligible. When the negative control was used to correct the data, estimates of the RBV derived from Hb gain were similar to those derived from the HRE. These results suggest that cocoa is a significant source of moderately bioavailable Fe.

Fe bioavailability: Cocoa powder: Rats: Hb regeneration efficiency method

Fe deficiency is one of the most important nutritional problems in the world today[1]. Many efforts are underway to develop effective measures for preventing Fe deficiency and anaemia[2]. Although chocolate drink powder and cocoa powder have been shown to be effective vehicles for dietary Fe fortification[3–5], the bioavailability of Fe naturally occurring in cocoa has not been quantified. Cocoa powders contain high amounts of Fe[6] and polyphenols, including epicatechin, catechin and their polymers (tannin)[7], and phytate[8]. Polyphenols and phytate are potent inhibitors of Fe absorption[9,10]. Consistent with this, our previous studies showed that frequent consumption of tea (a source of tannin) and bran (a source of phytate) is associated with low-Fe stores in women[11,12]. The high level of polyphenols and phytate in cocoa powder may decrease the Fe bioavailability. However, fermentation of phytate-rich plant foods increases the Fe bioavailability[13,14]. To produce cocoa powder, cocoa beans are cured or fermented, dried and roasted before grinding[15], which may increase the Fe bioavailability. The aim of the present study was to quantify the Fe bioavailability of cocoa powders using the Hb regeneration efficiency (HRE) method[16] with minor modifications.

Materials and methods

The Fe bioavailability of cocoa powder was determined using the HRE method[16,17] and thirty-five weanling F/344N male

rats (Japan SLC, Shizuoka, Japan), with a mean weight of 50·4 g (SE 0·7 g). At the start of the study, each animal was weighed, blood was collected by tail-vein puncture and the Hb concentration of fresh blood samples was measured using the cyanmethaemoglobin method and an electronic counter (Cellutac MEK-5258, Nihon Kohden Co., Tokyo, Japan). The animals were individually housed in stainless steel cages with raised wire bases and maintained in a temperature- and light-controlled environment at 22°C and 50 % relative humidity with a standard light cycle (12 h light/12 h dark; lights on 07.00 hours). All the animals had free access to the experimental diets and deionised water during the entire periods.

Anaemia was induced in all animals by feeding them a low-Fe diet (3·6 mg Fe/kg) for 4 weeks. The diet consisted of an AIN-93G-purified rat diet without ferric citrate and contained (g/kg): maize starch, 397·486; casein, 200·000; dextrinised maize starch, 132·000; sucrose, 100·000; soyabean oil, 70·000; fibre (cellulose powder), 50·000; AIN-93G mineral mix (without ferric citrate), 35·000; AIN-93 vitamin mix, 10·000; L-cystine, 3·000; choline bitartrate, 2·500; *tert*-butylhydroquinone, 0·014. Casein, maize starch, fibre, AIN-93G mineral mix (without ferric citrate) and AIN-93 vitamin mix were obtained from the Oriental Yeast Japan Co. (Tokyo, Japan). Dextrinised maize starch was obtained from Nacalai Tesque Co. (Kyoto, Japan). Other ingredients were obtained from Wako Pure Chemicals (Osaka, Japan).

After the 28-d depletion period, each animal was weighed, blood was collected by tail-vein puncture and the Hb concentration was measured. The animals were then randomised into five groups (of seven rats each) based on the Hb concentrations.

The AIN-93G diet uses 35 mg Fe/kg in the form of ferric citrate, which has a lower Fe bioavailability than ferrous sulphate[18]. The dose–response curve for ferrous sulphate is linear up to 24 mg Fe/kg according to the Association of Official Agricultural Chemists method[19]. Therefore, we used repletion diets containing 20 mg Fe/kg to estimate the Fe bioavailability. Four repletion diets were prepared by replacing the maize starch component of the low-Fe diet with one of two brands of commercial cocoa powder (Cocoa A (Morinaga Brand Pure Cocoa; Morinaga & Co. Ltd, Tokyo, Japan) and Cocoa B (Van Houten Brand Pure Cocoa; Kataoka & Co. Ltd, Tokyo, Japan)), ferrous sulphate or ferric citrate (Wako Pure Chemicals). The cocoa powders were purchased from a local supermarket in Matsudo, Chiba, Japan. Cocoa A and Cocoa B contained 30·39 and 29·43 mg Fe/100 g, respectively. Therefore, the cocoa powder repletion diets contained 65·81 g/kg of Cocoa A or 67·95 g/kg of Cocoa B. Each repletion diet was fed to one of the four groups of seven animals (total twenty-eight animals) during the 20-d repletion period. The other seven rats were maintained on the low-Fe diet during this period as a negative control.

The cocoa powders, diets and a National Institute of Standards and Technology (Gaithersburg, MD, USA) standard reference material 1548a typical diet were wet-ashed using concentrated HNO_3 and 30 % H_2O_2 (Wako Pure Chemicals) with appropriate heating[20]. The decomposed samples were diluted with 1 % (w/v) HNO_3 and the Fe concentrations were measured using inductively coupled plasma-MS (ICPM-8500, Shimadzu Co., Kyoto, Japan). The analysed value of the National Institute of Standards and Technology standard reference material 1548a typical diet was 34·5 mg Fe/g, whereas the value certified by the National Institute of Standards and Technology was 35·3 mg Fe/g with a 95 % CI of 3·77 mg/g.

The apparent feed consumption of each animal was recorded during the experimental period. At the end of the repletion period, body weight, Hb concentration and total feed intake were determined. The animals were then euthanised by exsanguination under diethyl ether anaesthesia.

Hb-Fe (mg) was calculated[16,21] as follows:

$$Hb\text{-}Fe\,(mg) = body\,weight\,(kg) \times \frac{0\cdot075\,litres\,blood}{body\,weight\,(kg)}$$

$$\times \frac{Hb\,(g)}{blood\,(l)} \times \frac{3\cdot35\,mg\,Fe}{Hb\,(g)}.$$

The HRE values were calculated using the following equation:

$$HRE = \frac{(mg\,Hb\text{-}Fe\,(final) - mg\,Hb\text{-}Fe\,(initial))}{mg\,Fe\,consumed}.$$

The relative biological value (RBV) based on the HRE (RBV_{HRE}) was calculated by dividing the individual HRE values of the Fe sources by the mean HRE value of ferrous sulphate.

$$RBV_{HRE} = \frac{HRE\,(each\,animal)}{HRE\,(average\,for\,ferrous\,sulphate)}.$$

We also calculated the RBV based on the Hb gain ($RBV_{Hb\text{-}gain}$) for a comparison with the RBV_{HRE}, as recommended by Forbes *et al.*[16]. The $RBV_{Hb\text{-}gain}$ was calculated by dividing the individual Hb gains of the Fe sources by the mean Hb gain of ferrous sulphate.

The Hb gain was calculated for each animal according to the following equation:

$$Hb\text{-}gain = Hb\,(final) - Hb\,(initial).$$

The $RBV_{Hb\text{-}gain}$ was calculated for each animal according to the following equation:

$$RBV_{Hb\text{-}gain} = \frac{Hb\text{-}gain\,(each\,animal)}{Hb\text{-}gain\,(average\,for\,ferrous\,sulphate)}.$$

Since the Hb concentration of rats fed the low-Fe diet continued to decrease during the repletion period, the mean Hb gain for the low-Fe diet was subtracted from the respective Hb gains to obtain a corrected $RBV_{Hb\text{-}gain}$.

The Hb gain was corrected as follows:

$$Corrected\,Hb\text{-}gain = Hb\text{-}gain\,(each\,animal) - Hb\text{-}gain$$

$$(average\,for\,the\,negative\,control).$$

Then, the corrected $RBV_{Hb\text{-}gain}$ was calculated as follows:

$$Corrected\,RBV_{Hb\text{-}gain}$$

$$= \frac{Corrected\,Hb\text{-}gain\,(each\,animal)}{Corrected\,Hb\text{-}gain\,(average\,for\,ferrous\,sulphate)}.$$

The data were analysed by Scheffé's simultaneous multiple comparison test and Spearman's rank correlation test using SYSTAT version 10.2 software (SPSS Inc., Evanston, IL, USA) for Microsoft Windows. *P* values less than 0·05 were considered significant.

The present study was approved by the Ethical Committee for Laboratory Animals of Seitoku University, Matsudo, Chiba, Japan. All animal care guideline and animal handling procedures followed were concordant with the standards relating to the Care and Management of Experimental Animals (notification no. 6, 1980, The Japanese Prime Minister's Office).

Results

At the start of the depletion period, the mean Hb concentration was 123 (SE 1) g/l and mean body weight was 50·4 g (SE 0·7 g). At the end of the depletion period, the mean Hb concentration was 64 (SE 1) g/l and mean body weight was 130·6 g (SE 1·3 g). Body weights, and body-weight gains, feed intakes, feed efficiencies and Fe intakes during the repletion period are shown in Table 1. The mean Hb gain of the negative control was −11 (SE 1) g/l. The mean Hb-Fe gain of the negative control was 0·01 mg (SE 0·03 mg), signifying that the dietary treatment was effective in depleting Fe. Although the initial body weight of rats that were fed Cocoa A was slightly higher than that of the other groups, the differences were

Table 1. Body-weight gain, feed intake, feed efficiency and Fe intake of rats during Fe repletion

(Mean values with their standard errors)

	Negative control		Ferrous sulphate		Ferric citrate		Cocoa A*		Cocoa B†		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	ANOVA P value
Body weight (g)											
Initial	132·3	2·5	126·9	2·7	129·0	2·7	136·2	1·7	128·5	4·3	0·189
Gain	28·7[a]	2·0	78·4[b]	3·6	57·2[c]	2·2	67·0[bc]	1·6	60·0[c]	4·2	<0·001
Feed intake (g)	177·9[a]	4·1	257·1[b]	8·9	216·7[c]	5·1	259·2[b]	3·7	244·0[b,c]	10·3	<0·001
Feed efficiency	0·162[a]	0·011	0·304[b]	0·004	0·264[c]	0·008	0·259[c]	0·006	0·245[c]	0·008	<0·001
Fe intake (mg)	0·64[a]	0·01	6·07[b]	0·21	5·12[c]	0·12	6·12[b]	0·09	5·76[bc]	0·24	<0·001

[a,b] Mean values within a row with unlike superscript letters significantly differ ($P<0.05$) according to Scheffé's multiple comparison test.
* Morinaga Brand Pure Cocoa (Morinaga & Co., Tokyo, Japan).
† Van Houten Brand Pure Cocoa (Kataoka & Co., Tokyo, Japan).

NS. This was probably an artifact because the rats were allocated to treatments based on the Hb concentration alone. The initial Hb concentrations were similar between groups.

The RBV of the commercially available cocoa powders and ferric citrate were compared with that of ferrous sulphate (Table 2). All indicators of the Fe bioavailability, i.e. Hb gain, Hb-Fe gain, HRE and RBV of cocoa powders and ferric citrate, were significantly lower than that of ferrous sulphate. These indicators revealed that the Fe bioavailability of the cocoa powders was almost identical to that of ferric citrate and that the difference between the two brands of cocoa powder was negligible.

The relationship between the Hb gain and the body-weight gain and that between the Hb gain and the feed intake are shown in Fig. 1. The body-weight gain was significantly correlated with the Hb gain and feed intake during the repletion period (Spearman's $\rho = 0.846$, n 35, $P<0.001$; Spearman's $\rho = 0.880$, n 35, $P<0.001$, respectively). The feed intake was significantly correlated with the Hb gain (Spearman's $\rho = 0.714$, n 35, $P<0.001$).

Discussion

According to the International Cocoa Organization, the revised estimate of world cacao bean grindings for the 2006–2007 cocoa year was 3 641 000 tons[22]. Cocoa grinding per capita per year in 2005 was approximately 2000 g in the UK and 400 g in Japan. According to the United States Department of Agriculture, Economic Research Service food availability data, chocolate-liquor disappearance (i.e. total use minus exports) in the USA was 5·25 pounds/year per capita, which corresponds to approximately 3·3 g cocoa powder/d per capita, assuming that chocolate liquor contains 50 % cocoa powder on the weight basis[23]. The Fe content in cocoa powder is approximately 15–40 mg/100 g according to the USDA National Nutrient Database for Standard Reference (United States Department of Agriculture, Agricultural Research Service, Nutrient Data Laboratory). Therefore, 6 g of cocoa powder is equivalent to 0·9–2·4 mg Fe. This amount of Fe would be nutritionally significant, if the Fe in cocoa powder were bioavailable. Buijsse et al.[24] investigated the relationship between the cocoa intake and blood pressure among elderly men in The Netherlands. They found that one-third of the population did not consume cocoa powder and that the median and the 92nd percentile intakes of cocoa powder from various sources were 0·92 and 6·1 g/d, respectively.

Considering measurement errors, the RBV as determined by the modified HRE method was similar to that for ferric citrate and the two brands of cocoa powder, and the difference between the two brands of cocoa powder was negligible. The Fe bioavailability of cocoa is similar to that of lime-treated

Table 2. Bioavailability for rats of ferric citrate and Fe in commercial cocoa powder using the AIN-93G diet

(Mean values with their standard errors)

	Negative control		Ferrous sulphate		Ferric citrate		Cocoa A*		Cocoa B†		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	ANOVA P value
Hb (g/l)											
Initial	63	1	63	2	64	2	64	2	64	2	0·999
Gain	−11[a]	0·4	62[b]	3	19[c]	2	22[c]	1	21[c]	3	<0·001
Hb-Fe (mg)											
Initial	2·11	0·07	2·01	0·05	2·06	0·09	2·18	0·08	2·06	0·10	0·490
Gain	0·01[a]	0·03	4·45[b]	0·21	1·79[c]	0·13	2·19[c]	0·10	1·96[c]	0·21	<0·001
HRE	0·016[a]	0·040	0·733[b]	0·025	0·350[c]	0·023	0·357[c]	0·013	0·336[c]	0·026	<0·001
RBV_HRE	0·022[a]	0·055	1·000[b]	0·034	0·478[c]	0·032	0·488[c]	0·018	0·459[c]	0·036	<0·001
RBV_Hb-gain	−0·177[a]	0·007	1·000[b]	0·046	0·302[c]	0·037	0·352[c]	0·022	0·338[c]	0·059	<0·001
Corrected RBV_Hb-gain	0·000[a]	0·006	1·000[b]	0·039	0·407[c]	0·032	0·450[c]	0·019	0·438[c]	0·043	<0·001

HRE, Hb regeneration efficiency; RBV_HRE, relative biological value based on HRE; RBV_HRE, relative biological value based on Hb gain.
[a,b,c] Mean values within a row with unlike superscript letters significantly differ ($P<0.05$) according to Scheffé's multiple comparison test.
* Morinaga Brand Pure Cocoa (Morinaga & Co., Tokyo, Japan).
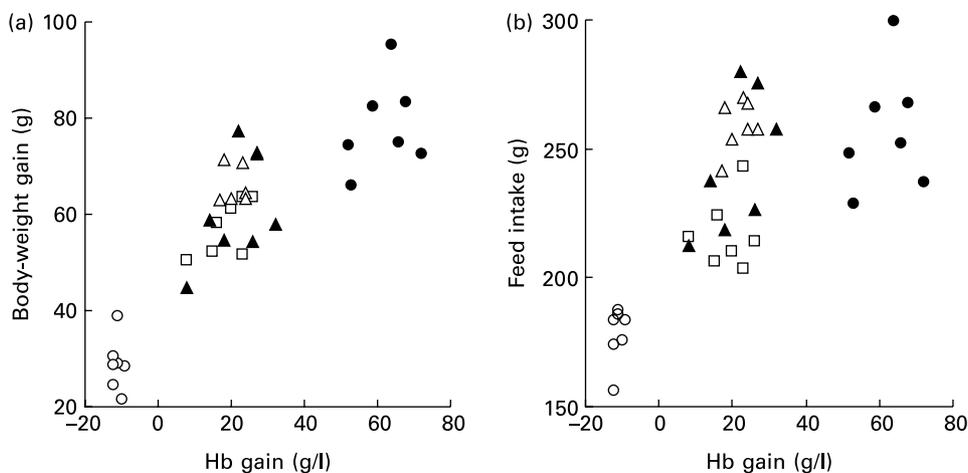† Van Houten Brand Pure Cocoa (Kataoka & Co., Tokyo, Japan).

Fig. 1. Relationship between Hb gain, body-weight gain and feed intake during the repletion period. (a) The relationship between Hb gain and body-weight gain (Spearman's $\rho = 0.846$, n 35, $P < 0.001$). (b) The relationship between Hb gain and feed intake (Spearman's $\rho = 0.714$, n 35, $P < 0.001$). The symbols indicate the data for individual rats: ○, Fe-deficient diet; ●, ferrous sulphate diet; □, ferric citrate diet; △, Cocoa A diet; ▲, Cocoa B diet.

maize flour (RBV 0.51) and wheat flour with 70–72 % extraction (RBV 0.55), as determined using the HRE method by Hernández *et al.* [25], and is better than that of ferric phosphate (RBV 0.23) reported by Whittaker & Vanderveen [26]. These results indicate that the Fe in cocoa is moderately bioavailable and would be a significant Fe source if the results of animal studies were applicable to human subjects.

Cocoa contains considerable amounts of Fe absorption inhibitors. According to Natsume *et al.* [27], the total polyphenol or tannin content of pure cocoa is 3.02–4.73 %. Aremu *et al.* [8] reported that five brands of Nigerian cocoa beverage contained 68–146 mg of oxalate and 590–750 mg of phytate per 100 g of DM. On the other hand, cocoa is a fermented food. During fermentation by yeasts, lactic acid bacteria and acetobacters, significant amounts of polyphenols and alkaloids in cacao beans are metabolised and lost [28]. Effective yeast fermentation, which degrades phytate, increases the bioavailability of Fe in whole-meal bread [13,29]. Further studies are necessary to determine whether cocoa Fe bioavailability in human subjects is similar to that in rodents.

In addition to information on the bioavailability of cocoa Fe, the present results also emphasise the significance of methodological aspects of the HRE method. The International Nutritional Anemia Consultative Group Task Force reported that the RBV values derived using the HRE method are greater than those derived using the modified Association of Official Agricultural Chemists method or the Hb gain method, especially for lower RBV or less bioavailable compounds [16]. The task force mentioned the shorter depletion period and the compensation of the body-weight-gain factor as possible causes of the apparent overestimates of the RBV of less bioavailable compounds.

In the present study, we modified the HRE method of Forbes *et al.* [16] by extending the depletion period from 7–8 to 28 d and the repletion period from 9–10 to 20 d, and calculated the RBV based on the Hb gain method for comparison. However, we did not check the RBV using the Association of Official Agricultural Chemists method. The RBV_{HRE} was significantly greater than the $RBV_{Hb\text{-gain}}$ for ferric citrate and Cocoa A ($P < 0.01$ according to Scheffé's simultaneous comparison test). By contrast, there were no significant

differences between the RBV_{HRE} and the corrected $RBV_{Hb\text{-gain}}$ for all Fe sources. The Fe deficiency decreases the body-weight gain and the feed intake in rats [30–32]. Concordant with this, there were strong positive correlations between the Hb gain, the body-weight gain and the feed intake (Fig. 1; Table 1). Ferric citrate resulted in the smallest Hb gain, body-weight gain and feed intake among the materials tested (excluding the negative control). We assume that the slightly lower bioavailability of ferric citrate according to the $RBV_{Hb\text{-gain}}$ may have contributed to the lower feed intake and body-weight gain of rats fed this compound. The Hb gain, body-weight gain and feed intake are used to calculate the RBV_{HRE}. We suspect that the overestimation of the RBV of low bioavailable compounds by the HRE method compared with the Hb gain method is an inherent feature of the formula used to calculate the RBV_{HRE} (Appendix).

During the depletion period, the negative control rats either gained Hb-Fe or did not lose Hb-Fe. They gained body weight, whereas their Hb level decreased reciprocally. We believe that the zero-point correction of the $RBV_{Hb\text{-gain}}$ using the negative control data is an effective means of avoiding underestimates of the RBV by the Hb gain method relative to the estimates derived using the HRE method. In the present study, the Hb gain-derived RBV and the HRE-derived RBV resulted in similar estimates of the Fe bioavailability for the various Fe sources. However, there were insufficient data to establish whether data correction using the negative control data is relevant to the conditions in which depletion is more severe or in which shorter depletion and repletion periods are applied.

In conclusion, these results suggest that the Fe bioavailability of cocoa powder as determined using a rodent model is moderate and similar to that of ferric citrate. Cocoa powders may be significant Fe sources for human subjects.

Acknowledgements

analysis and contributed to the drafting and the writing of the manuscript; A. K. contributed to the execution of the experiment and to the drafting of the manuscript; M. O. contributed to the execution of the experiment and to the data collection.

References

1. World Health Organization (2008) World prevalence of anaemia 1993–2005. In *WHO Global Database on Anaemia*, [De Benoist B, Mclean E, Egli I & Cogswell M, editors]. Geneva: World Health Organization.
2. Konomi A & Yokoi K (2005) Zinc deficiency decreases plasma erythropoietin concentration in rats. *Biol Trace Elem Res* **107**, 289–292.
3. Navas-Carretero S, Sarria B, Perez-Granados AM, *et al.* (2007) A comparative study of iron bioavailability from cocoa supplemented with ferric pyrophosphate or ferrous fumarate in rats. *Ann Nutr Metab* **51**, 204–207.
4. Fidler MC, Walczyk T, Davidsson L, *et al.* (2004) A micronised, dispersible ferric pyrophosphate with high relative bioavailability in man. *Br J Nutr* **91**, 107–112.
5. Hurrell RF, Reddy MB, Dassenko SA, *et al.* (1991) Ferrous fumarate fortification of a chocolate drink powder. *Br J Nutr* **65**, 271–283.
6. Johnston FA & Collier A (1950) Iron content of cocoa, baking chocolate, and milk chocolate. *J Am Diet Assoc* **26**, 93.
7. Roura E, Andres-Lacueva C, Estruch R, *et al.* (2006) Total polyphenol intake estimated by a modified Folin–Ciocalteu assay of urine. *Clin Chem* **52**, 749–752.
8. Aremu CY & Abara AE (1992) Hydrocyanate, oxalate, phytate, calcium and zinc in selected brands of Nigerian cocoa beverage. *Plant Foods Hum Nutr* **42**, 231–237.
9. Sharpe LM, Peacock WC, Cooke R, *et al.* (1950) The effect of phytate and other food factors on iron absorption. *J Nutr* **41**, 433–446.
10. Sandberg AS (2002) Bioavailability of minerals in legumes. *Br J Nutr* **88**, Suppl. 3, S281–S285.
11. Yokoi K, Alcock NW & Sandstead HH (1994) Iron and zinc nutriture of premenopausal women: associations of diet with serum ferritin and plasma zinc disappearance and of serum ferritin with plasma zinc and plasma zinc disappearance. *J Lab Clin Med* **124**, 852–861.
12. Yokoi K, Sandstead HH, Egger NG, *et al.* (2007) Association between zinc pool sizes and iron stores in premenopausal women without anaemia. *Br J Nutr* **98**, 1214–1223.
13. Sandberg AS, Brune M, Carlsson NG, *et al.* (1999) Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. *Am J Clin Nutr* **70**, 240–246.
14. Baynes RD, Macfarlane BJ, Bothwell TH, *et al.* (1990) The promotive effect of soy sauce on iron absorption in human subjects. *Eur J Clin Nutr* **44**, 419–424.
15. Morgan J (1994) Chocolate: a flavor and texture unlike any other. *Am J Clin Nutr* **60**, 1065S–1067S.
16. Forbes AL, Arnaud MJ, Chichester CO, *et al.* (1989) Comparison of *in vitro*, animal, and clinical determinations of iron bioavailability: international nutritional anemia consultative group task force report on iron bioavailability. *Am J Clin Nutr* **49**, 225–238.
17. Mahoney AW & Hendricks DG (1982) Efficiency of hemoglobin regeneration as a method of assessing iron bioavailability in food products. In *Nutritional Bioavailability of Iron*, pp. 1–11 [C Kies, editor]. Washington, DC: American Chemical Society.
18. Kwock RO, Keen CL, Hegenauer J, *et al.* (1984) Retention and distribution of iron added to cow's milk and human milk as various salts and chelates. *J Nutr* **114**, 1454–1461.
19. Swain JH, Newman SM & Hunt JR (2003) Bioavailability of elemental iron powders to rats is less than bakery-grade ferrous sulfate and predicted by iron solubility and particle surface area. *J Nutr* **133**, 3546–3552.
20. Yokoi K, Kawaai T, Konomi A, *et al.* (2008) Dermal absorption of inorganic germanium. *Regul Toxicol Pharmacol* **52**, 169–173.
21. Whittaker P, Mahoney AW & Hendricks DG (1984) Effect of iron-deficiency anemia on percent blood volume in growing rats. *J Nutr* **114**, 1137–1142.
22. International Cocoa Organization (2008) *Quarterly Bulletin of Cocoa Statistics, Vol. 33, No. 4, Cocoa Year 2006/07.* London: International Cocoa Organization.
23. United States Department of Agriculture, Economic Research Service (2008) Food availability: Spreadsheets, cocoa – supply and disappearance, http://www.ers.usda.gov/data/foodconsumption/foodavailspreadsheets.htm (27 August 2008).
24. Buijsse B, Feskens EJ, Kok FJ, *et al.* (2006) Cocoa intake, blood pressure, and cardiovascular mortality: The Zutphen Elderly Study. *Arch Intern Med* **166**, 411–417.
25. Hernández M, Sousa V, Moreno A, *et al.* (2003) Iron bioavailability and utilization in rats are lower from lime-treated corn flour than from wheat flour when they are fortified with different sources of iron. *J Nutr* **133**, 154–159.
26. Whittaker P & Vanderveen JE (1990) Effect of EDTA on the bioavailability to rats of fortification iron used in Egyptian balady bread. *Br J Nutr* **63**, 587–595.
27. Natsume M, Osakabe N, Yamagishi M, *et al.* (2000) Analyses of polyphenols in cacao liquor, cocoa, and chocolate by normal-phase and reversed-phase HPLC. *Biosci Biotechnol Biochem* **64**, 2581–2587.
28. Camu N, Gonzalez A, De Winter T, *et al.* (2008) Influence of turning and environmental contamination on the dynamics of populations of lactic acid and acetic acid bacteria involved in spontaneous cocoa bean heap fermentation in Ghana. *Appl Environ Microbiol* **74**, 86–98.
29. Brune M, Rossander-Hultén L, Hallberg L, *et al.* (1992) Iron absorption from bread in humans: inhibiting effects of cereal fiber, phytate and inositol phosphates with different numbers of phosphate groups. *J Nutr* **122**, 442–449.
30. Yokoi K, Kimura M & Itokawa Y (1991) Effect of dietary iron deficiency on mineral levels in tissues of rats. *Biol Trace Elem Res* **29**, 257–265.
31. Stangl GI & Kirchgessner M (1998) Effect of different degrees of moderate iron deficiency on the activities of tricarboxylic acid cycle enzymes, and the cytochrome oxidase, and the iron, copper, and zinc concentrations in rat tissues. *Z Ernahrungswiss* **37**, 260–268.
32. Beard JL, Zhan CS & Brigham DE (1995) Growth in iron-deficient rats. *Proc Soc Exp Biol Med* **209**, 65–72.

Appendix

Hb gain, feed intake and body-weight gain during the repletion period are highly correlated in rats used for estimating the relative biological values of various Fe sources by the Hb regeneration efficiency method. Although the assumption of exact proportionality between these measures is somewhat of an oversimplification, it is useful for unveiling the hidden relationship between the RBV_{HRE} and the $RBV_{Hb\text{-}gain}$.

Assuming an exact proportionality between the Hb gain, the feed intake and the body-weight gain, i.e. $Hb_g = m\,FI$ and $BW_g = n\,FI$, HRE becomes

$$HRE = \frac{b\,Hb_f BW_f - b\,Hb_i BW_i}{a\,FI},$$

where FI is the feed intake; a is the Fe content of feed; b is the Fe content of Hb multiplied by blood volume per unit body weight; BW_i is the initial body weight; BW_f is the final body weight;

BW$_g$ is the body-weight gain; Hb$_i$ is the initial Hb concentration; Hb$_f$ is the final Hb concentration; Hb$_g$ is the Hb gain; and m and n are the slopes of the regression equations.

Substituting Hb$_f$ with Hb$_i$ + Hb$_g$,

$$= \frac{b}{a} \left(\frac{(\text{Hb}_g + \text{Hb}_i)\text{BW}_f - \text{Hb}_i\text{BW}_i}{\text{FI}} \right)$$

$$= \frac{b}{a} \left(\frac{\text{Hb}_g\,\text{BW}_f + \text{Hb}_i\,\text{BW}_f - \text{Hb}_i\,\text{BW}_i}{\text{FI}} \right)$$

$$= \frac{b}{a} \left(\frac{\text{Hb}_g\,\text{BW}_f + \text{Hb}_i\,(\text{BW}_f - \text{BW}_i)}{\text{FI}} \right)$$

Substituting BW$_f$ with BW$_i$ + BW$_g$,

$$= \frac{b}{a} \left(\frac{\text{Hb}_g\text{BW}_f + \text{Hb}_i\text{BW}_g}{\text{FI}} \right)$$

$$= \frac{b}{a} \left(\frac{\text{Hb}_g}{\text{FI}} \right) \text{BW}_f + \frac{b}{a}\text{Hb}_i \left(\frac{\text{BW}_g}{\text{FI}} \right)$$

Substituting Hb$_g$ with m FI and BW$_g$ with n FI,

$$= \frac{b}{a} \left(\frac{m\,\text{FI}}{\text{FI}} \right) \text{BW}_f + \frac{b}{a}\text{Hb}_i \left(\frac{n\,\text{FI}}{\text{FI}} \right)$$

$$= \frac{b\,m}{a} \left(\text{BW}_g + \text{BW}_i \right) + \frac{b\,n}{a}\text{Hb}_i = \frac{b\,m}{a}(\text{n FI} + \text{BW}_i) + \frac{b\,n}{a}\text{Hb}_i$$

$$= \frac{b\,n}{a}\text{m FI} + \frac{b\,m}{a}\text{BW}_i + \frac{b\,n}{a}\text{Hb}_i$$

Substituting FI with Hb$_g$/m,

$$= \frac{b\,n}{a}\text{Hb}_g + \frac{b\,m}{a}\text{BW}_i + \frac{b\,n}{a}\text{Hb}_i$$

$$= \frac{b\,n}{a}\text{Hb}_g + \frac{b}{a}(m\,\text{BW}_i + n\,\text{Hb}_i)$$

Since BW$_i$ and Hb$_i$ are equalised among rats fed different Fe sources, the second term can be replaced by the constant C,

$$\text{HRE} = \frac{b\,n}{a}\text{Hb}_g + C$$

Therefore, the discrepancy between the RBV$_{\text{HRE}}$ and the RBV$_{\text{Hb-gain}}$ is larger for compounds with low-Fe bioavailability because the contribution of the constant C to HRE is relatively larger at a low Hb$_g$.