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Methods and Options for *in vitro* Dialyzability; Benefits and Limitations

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Abstract: *In vitro* dialyzability methods involve a two-step digestion process simulating the gastric and intestinal phase, and dialysis through a semi-permeable membrane with a selected molecular weight cut-off. Dialyzable iron/zinc is used as an estimation of available mineral. Final pH adjustment and use of a strict time schedule were found to be critical factors for standardization. In addition the selected cut-off of the dialysis membrane and the method used for iron and zinc determination influence the results.

For screening purposes, simple solubility or dialyzability methods seem preferable to the more sophisticated computer-controlled gastrointestinal model. This is likely more valuable in studies of different transit times and sites of dialyzability.

In vitro solubility/dialyzability methods correlate in most cases with human absorption studies in ranking iron and zinc availability from different meals. Exceptions may be that effects of milk, certain proteins, tea, and organic acids cannot be predicted. The dialyzability methods exclude iron bound to large molecules, which in some cases is available and include iron bound to small molecules, which is not always available.

In vitro experiments based on solubility/dialyzability are tools to understand factors that may affect subsequent mineral absorption.

Key words: *In vitro,* iron availability, dialyzability, zinc availability

Background

Human studies are time-consuming, expensive, and complicated to perform. The need for the development of animal models and *in vitro* methods for screening purposes is therefore obvious. Animal models have certain limitations because there are differences in mineral requirements, metabolism, digestive capacity, and sensitivity to dietary factors compared to humans. Rats, for example, are of limited value for assessing iron absorption in humans [1].

The *in vitro* methods are faster, less expensive, and should offer better control of experimental variables. However, it is of utmost importance that *in vitro* methods are validated; i.e. *in vivo* measurements of absorption from the same foods and diets are performed and a high degree of correlation is obtained.

In vitro methods are designed to simulate the *in vivo* environment and the choice of conditions are thus based on knowledge of gastrointestinal physiology and the ability to mimic this. One approach which will be discussed in this paper is the *in vitro* dialyzability method, which was introduced by Miller *et al* [2] This method involve a two-step digestion phase simulating the gastric and intestinal phase and dialysis through a semi-permeable membrane with a selected molecular-weight cut-off. Di-

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alyzable iron/zinc is then used as an estimation of available mineral.

Limitations with *in vitro* methods

Generally there are a number of limitations with the use of *in vitro* methods because the interactions between the digestive system and the food ingested are not measured (see [3–5] for review). Factors such as transit time, site of absorption, composition of digestive secretions responding to the meal, and intestinal flora may all affect the mineral availability. These interactions are summarized in Table I.

These physiological interactions are impossible to factor in *in vitro* work. Furthermore, because active uptake of iron is not measured by *in vitro* methods for soluble/dialyzable iron, the effect of mineral status cannot be measured and heme iron availability is not measured by *in vitro* solubility/dialyzability.

Despite the complexity, some of the physicochemical factors affecting mineral bioavailability would be possible to simulate *in vitro*. Some of these factors have been listed by Johnson [3], Table II.

The minerals that are solubilized in the stomach and remain soluble in the duodenum must still diffuse to the surface of the brush border membrane before they can be absorbed by the mucosal cell. It is likely that the rate of diffusion of minerals to the brush border will influence the extent to which they are absorbed. The mucus gel is considered to be permeable to small ions, and it has been suggested that one function of the adherent mucus layer may be to exclude macromolecules from reaching the absorptive surface of mucosal cells [6, 7]. Thus, soluble nutrients bound to macromolecules will presumably be absorbed at a lower rate than nutrients present in a low-molecular weight form. This diffusion step is intended to be simulated *in vitro* by the use of dialysis.

Basis for dialyzable iron methods

Iron is taken as an example because most studies in the literature are performed on iron. Only a fraction of soluble iron is bioavailable and insoluble iron is not absorbed. Ferric iron is easily hydrolyzed at a pH > 1, leading to formation of iron hydroxides, which usually renders iron insoluble. Thus, iron must be kept in the ferrous form or bound to ligands to keep it soluble (Figure 1).

The available iron (bioaccessible) is defined as the fraction of the total iron that is available for uptake by the intestinal brush border membranes. Iron absorption involves several steps: i) digestion and release of iron from the diet, ii) diffusion of iron to the brush border, iii) active uptake of iron into the enterocytes, and iv) transport from the enterocytes to the circulation. Most food factors influence iron before absorption. The two first steps are intended to be simulated with the *in vitro* dialyzable iron methods.

Early *in vitro* methods developed for estimation of iron availability were based on digestion simulating the gastric phase using incubation with pepsin and HCl and mea-

Table II: Physico-chemical factors affecting mineral bioavailability [3]

Concentration/dose
pH
Chelation
Solubility
Molecular weight of complexes
Structure of ligand/receptors
Non-digestible food components – fiber
Oxidation state
Micelles
Interactions with other minerals – multiple equilibria
Physical form of food

Table I: Limitations with *in vitro* methods

The interactions between the digestive system and the food ingested are not measured:
– The transit time through the digestive tract depends on the composition of the meal [39–42].
– Different parts of the digestive tract (duodenum, ileum, and colon) have different absorptive activities.
– The composition of digestive secretions (gastric, biliary, and pancreatic) responds to the meal eaten [43–48]. It is dependent on the composition of the meal, the consistency of the food, and its organoleptic properties.
– Intestinal flora is lacking, which may influence nutrient availability; e.g., some microorganisms produce siderophores or "iron carriers" to accumulate iron [49].
– Active uptake of minerals is not measured with *in vitro* methods for soluble/dialyzable minerals.
– Effect of mineral status cannot be measured.
– Heme iron availability is not measured by *in vitro* solubility/dialyzability.

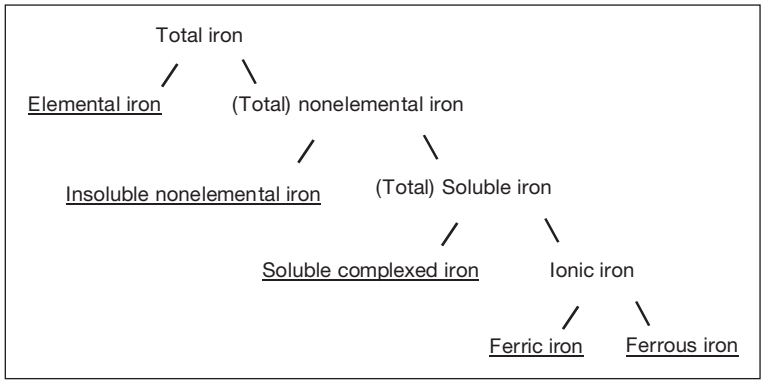

Figure 1: Chemical iron profile in foods according to Lee and Clydesdale [56]

surement of the soluble and ionizable iron [8, 9]. Narasinga Rao and Prabhavati [10] added to this pepsin-HCl extraction step a pH adjustment to 7.5 with NaOH to simulate the intestinal phase and soluble and ionizable iron was determined in the filtered supernatant. This method was followed by modifications developing the intestinal step by adding bile acids, pancreatic enzymes and adjustment of pH with $NaHCO_3$ [11–14].

Miller *et al* introduced equilibrium dialysis for estimation of iron availability [2]. Their method was based on digestion at simulated physiological conditions of the stomach and small intestine and that the soluble and dialyzable iron is considered available for absorption, instead of measurement of the iron fraction present in the supernatant after centrifugation. According to Miller the soluble iron, with a molecular weight cut-off of the dialysis tube of 6–8000 Da, was determined by a colorimetric method with bathophenanthroline or radioisotope technique (^{59}Fe).

A number of modifications of this method have been used (see Table III) involving differences in intestinal pH adjustment, cut-off of the dialysis membrane, and methods for determination of iron. In the early methods iron was determined by different colorimetric methods [15, 16].

To improve the final pH adjustment and make it uniform, Wolfgor *et al* [17] suggested a modified procedure with calculation of HCl equivalents needed to adjust the pH to 6.5 with PIPES buffer. Atomic absorption spectroscopy (AAS) was used for the determination of iron. With this method the coefficient of variation of iron dialyzability in iron-fortified samples is usually low (7%).

A continuous dialysis method was introduced by Wolters *et al* [18] using a simple hollow fiber system. Di-

Table III: In vitro iron solubility/dialyzability after simulated digestion

Author	Method	Gastric pH	Intestinal pH	Dialysis Cut-off	Fe method
Narasinga Rao, and Prebnavati 1978 [10]	Total soluble and ionizable iron	1.35	7.5		Colorimetric
Madriaga *et al*, 1984 [11]			7.0	–	^{59}Fe, gamma counter
Sandberg *et al*, 1989 [12]; Svanberg *et al*, 1993 [26]	Total soluble	2	6.0	–	AAS
Miller *et al*, 1981 [2]	Soluble and dialyzable	2	7.5	6–8000	Colorimetric, ^{59}Fe, gamma counter
Hazel and Johnson, 1987 [50]		2	7.0	12000	^{59}Fe, gamma counter
Hurrell *et al*, 1988 [15]		2	7.5	6–8000	Colorimetric
Kapsokefalou and Miller, 1991 [16]		2	6.1–6.3		Colorimetric
Wolfgor *et al*, 2001 [17]		2	6.5	6–8000	AAS
Wolters *et al*, 1993 [18]	Continuous dialysis	2	6.7–7	12–14000	AAS
Larsson *et al*, 1997 [21]	(dynamic GI model dialyzable)	gradient	6.5–6.9	3–5000	AAS
Salovaara *et al*, 2003 [20]		gradient	6.5-6.9	3–5000	HPLC

alyzable components are continuously removed from the pancreatic digestion mixture.

A more sophisticated model, i.e. a computer-controlled dynamic gastrointestinal model for continuous dialysis, was developed by Minekus *et al* [19]. This model was used to study iron and phosphorus availability [20, 21].

An inter-laboratory study was performed to compare *in vitro* dialyzability of iron according to Miller *et al* [2]. Nine laboratories participated in the study [22]. Shrimps and three different meals: A) macaroni and cheese, B) tuna noodle, C) stuffed pepper, containing 16.4, 19.7, and 33.1 mg/kg of total iron, respectively, were compared. The inter-laboratory variation was significant. It was concluded that the *in vitro* methods need precise standardization. The main problems identified were: inter-laboratory reproducibility, final pH adjustment, and use of a strict time schedule, as the longer an iron source remains in a liquid system the more is dissolved and dialyzed.

The importance of the pH for iron solubility in the intestine can be illustrated by a study we conducted to investigate the effect of different inositol phosphates on iron solubility at simulated physiological conditions [12]. When adding different concentrations of inositol tri- and inositol tetraphosphate (0.5–10 µmol) to a white wheat roll and measuring the iron solubility after the simulated gastric and intestinal phase, adjustment of pH to 6.0, 6.5, and 7.0 gave significantly different results. Solubility of iron when 10 µmol inositol tetraphosphate was added was 50%, 35%, and 22%, respectively, when pH was adjusted to 6.0, 6.5, and 7.0. However, the solubility of iron was not affected by the pH in the interval between 6.0 and 7.0 when inositol hexa- and pentaphosphate were added.

According to Wolfgor *et al* [17], the best correlation between dialyzable iron and *in vivo* measurements was obtained at a pH between 6 and 7.

Computer-controlled gastrointestinal model

A dynamic computer-controlled gastrointestinal model was developed at TNO, the Netherlands, to simulate the digestive process in the stomach and small intestine [19]. The model monitors and controls all aspects of digestion – including peristaltic movements, transit time, pH, and the addition of digestive juices and enzymes. By means of a dialysis system, composed of semi-permeable hollow fiber membranes connected to the jejunal and ileal compartments, digested low molecular weight products that are potentially available for absorption can be collected and analyzed. The fixed flow of gastric and salivary secretions is a simplification of the variable secretion of liquids *in vivo*, caused by reflex and feedback mechanisms. Thus, the model does not respond to the food; it is programmed to simulate the physiological conditions, which results in reproducible experiments irrespective of characteristics of the food. The model consists of four sections representing the stomach, duodenum, jejunum, and ileum, connected by peristaltic valves, which determine the rate of transport of the food/digesta. Secretions of digestive juices and pH adjustment in each section are simulated according to physiological data from the literature. These functions are computer-controlled and the parameters defined in different protocols, which are based on the type of meal studied. For determination of availability of nutrients, products of digestion, water, and other small molecules are collected from the jejunal and ileal compartments by pumping dialysis liquids through the semi-permeable hollow fiber membrane units with a molecular cut-off of approximately 3–5000 Da. Availability is then calculated as percentage dialyzability. Absorption can thus not be measured with this model. Drawbacks with this model are that it is expensive, time-consuming, laborious, large volumes of dialysates are produced, and contamination with zinc and copper occurs.

So far the model has only been used in two published studies of iron availability, one study to estimate iron and phosphorus availability in cereals coupled with the effect of addition of phytase [21], and one study of the effect of different transit times on iron availability [20]. As expected, the addition of phytase to the meals improved the iron availability [21].The latter study combined the gastrointestinal model with uptake studies in Caco-2 cells. We used a high-performance liquid chromatography (HPLC) method developed in our laboratory to determine iron in the dialysates [23].This method is based on the formation of a mineral complex with PCDA (pyridine-2,6-dicarboxylic acid), post-column derivatization with 4-(2-pyridylazo) resorcinol (PAR), and detection by UV-VIS at 500 nm. Because of the large amount of dialysate collected from the gastrointestinal (GI) model from one test meal up to 6 L, it is important that the method for mineral determination has a high sensitivity. Our method has a detection limit of 5 ng/g, which is lower than that of the AAS method. Three test meals were evaluated, consisting of lactic acid-fermented vegetables with white (I) or whole meal bread (II), and of sourdough-fermented rye bread (III). Three transit times were tested (fast, medium, and slow). Iron dialyzability differed significantly between medium and slow transit for meal I, and between fast and medium transit time for meal III. For meal II, high in phytate, the iron dialyzability was low irrespective of transit time. The meals could be ranked with respect to iron dialyzability and uptake in the order I, II, III. The results suggest that prolonged transit time may improve iron availability [20].

Comparison between *in vitro* dialyzable/soluble Fe/Zn and absorption in humans

A number of studies have been performed to correlate *in vitro* data on availability for human iron and zinc absorption using the same foods and diets. These studies are summarized in Table V. Most of them show a high correlation, but it is possible that studies that did not show correlation were not published.

The mean results of the previously mentioned collaborative study by Luten *et al* [22] were compared to *in vivo* data [1]. They found that dialyzability from meals A, B, C, and D was similar to non-heme iron absorption in humans in both ranking and magnitude.

Trinidad *et al* [24] studied iron absorption from three types of Filipino meals using extrinsic labeling with ^{59}Fe.

Table IV: In vitro iron solubility at simulated physiological conditions (Svanberg *et al*, unpublished) compared with human iron absorption from single meals [27, 28]

Diet	% Fe solubility	% non-heme Fe absorption
Wheat rolls (no detectable phytate)	56	27
Wheat rolls (4 mg phytate P)	42	20.7
Wheat rolls (200 mg phytate P)	16	7.7
Wheat rolls (200 mg phytate P +100 mg ascorbic acid)	45	28.1
Wheat rolls (200 mg phytate P)	8	3.1
Wheat rolls + tannic acid (25 mg)	20	9.3
Red sorghum (whole), Porridge	9	3.4
Red sorghum (dehulled), Porridge	10.5	4.3
Red sorghum (dehulled), Porridge	8	2
Wheat + red sorgh. Bran, Porridge	6	1.7
Wheat + white sorgh. Bran, Porridge	12	3.4
Red sorgh. dehulled + 50 mg ascorbic acid, Porridge	18	6.3
Mixed diet (minced meat, mashed potatoes, green beans)	28*	12
Spaghetti + meat sauce	31*	19
Wheat rolls + hamburger meat	41*	18
Hamburger meat	48*	20
Latin American diet (chapatti, black beans, rice)	9	3.6
Maize porridge	8	4.1
Unknown	27	13.6
Unknown	42	22

* The figures for *in vitro* availability of iron represent only non-heme iron and were thus corrected for estimated heme-iron availability (heme-iron in the meat was analyzed and its availability estimated from previous studies).

Meal A contained rice and vegetables, meal B rice and cocoa, and meal C rice with chicken and pork. Except for meal B there was good agreement between *in vitro* estimation of iron availability (percentage ionizable iron at pH 7.0) and absorption in humans. The cocoa present in meal B was considered to interfere with the colorimetric determination of iron.

Hurrell *et al* [15] studied the addition of different protein sources to a liquid meal, corn meal, or bread meal and compared *in vitro* dialyzable iron according to a modification of Miller's method [2], and non-heme iron absorption from single meals labeled with ^{59}Fe and ^{55}Fe. The protein sources studied were bovine serum albumin (BSA), beef, BSA plus egg white, and hemoglobin. The bioavailability of non-heme iron from meals with BSA was found to be over-estimated with the *in vitro* method. However, if excluding the BSA meals, a high correlation ($r = 0.95$) was found between dialyzable iron and iron absorption.

According to Turnlund *et al* [25], soluble and ionizable iron increased 2–3 times when milk was added to a cereal-based meal. However, adding milk to cereal-based meals in humans showed no significant effect on iron absorption as measured by stable isotopes.

Iron solubility at simulated physiological conditions (not dialysis) according to Svanberg *et al* [26] was compared to human iron absorption from a number of single meals from studies of Hallberg *et al* [27]. Samples of the same foods and diets were received from Hallberg's laboratory and used in the *in vitro* experiments. The diets were bread meals, porridge meals, mixed diets, hamburger meals, and meals with added ascorbic, tannic acid, or phytic acid. (Table IV, Svanberg *et al*, unpublished results, [27, 28]). The diets used in the *in vitro* experiments were freeze-dried and stored at –20 °C. For the diets with addition of ascorbic acid, tannic acid, or phytic acid, these components were not freeze-dried but added separately in the *in vitro* experiments and similarly added to the meals in the *in vivo* studies. The other diets contained negligible amounts of ascorbic acid. A high correlation was found between *in vitro* solubility of iron at simulated physiological conditions and human iron absorption from single meals labeled with ^{55}Fe and ^{59}Fe (Figure 2, [29]).

The results of dialyzability from the TNO model or combined dialyzability and Caco-2 cell uptake have been compared to human absorption data from some meals. In an European Union (EU) project (FAIR CT 95-0193, Nutritional and functional value of pea protein, NUTRIPEA C1004-95), which we coordinated, pea and soy infant formulas were dephytinized by phytase treatment, and compared to non-dephytinized formula [30]. The results showed that pea protein formula had a higher iron dialyzability than the soy protein formula, and that dephytinization of the pea and soy protein formulas resulted in

Table V: Comparison of *in vivo* absorption *in vitro* soluble dialyzable iron and zinc from same foods and diets

Author	Year	Soluble dialyzable *in vitro* method	Study	*In vivo*/*in vitro* correlation
Narasinga Rao and Prahbavati [10]	1978	Ionizable	Fe, 6 vegetarian diets	r = 0.94
Schricker *et al* [51]	1981	Dialyzable	Fe, 7 mixed meals	+
Lynch *et al* (Abstr) [52]	1982	Soluble	Fe	+
Sandström and Almgren [38]	1988	Dialyzable	Zn, 20 composite meals	+ r = 0.94
Svanberg *et al* (unpublished)	1993	Soluble	Fe/20 composite meals	+ r = 0.97
Hurrell *et al* [15]	1988	Dialyzable	Fe, 6 meals added protein	+ r = 0.95
Forbes *et al* [53]	1989	Soluble, dialyzable	Inter-laboratory trial, Fe fortification, 1 meal	(+)
Turnlund *et al* [25]	1990	Ionizable, soluble	Fe, 3 cereal meals with/without milk	–
Luten *et al* [22]	1996	Dialyzable	Inter-laboratory trial, Fe 3 vegetarian meals	(+)
Chiplonkar *et al* [54]	1999	Dialyzable	Fe, Zn, 38 vegetarian meals	+ r = 0.96 Fe r = 0.92 Zn
Trinidad *et al* [24]	1983	Soluble, ionizable	Fe, 3 Filipino meals	(+)
Walter *et al* [55]	2004	Dialyzable	Fe, corn-masa tortilla and fortification Fe	+ r = 0.96

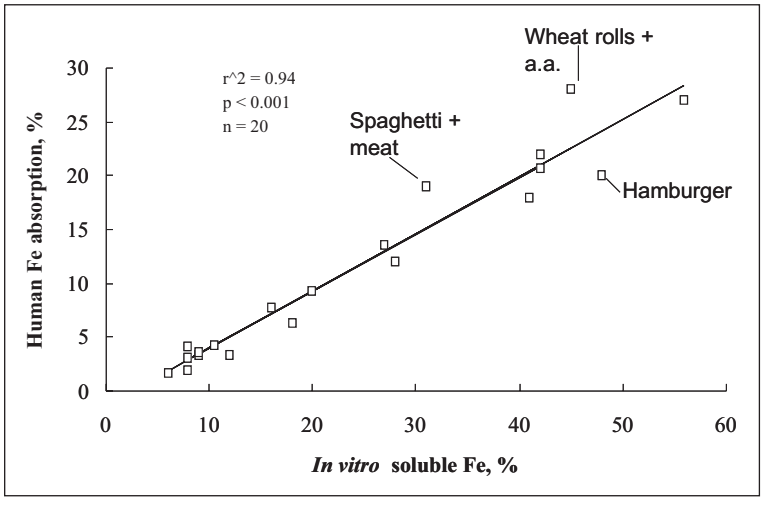

Figure 2: The relationship between human iron absorption from single meals labeled with ^{55}Fe and ^{59}Fe and *in vitro* estimation of soluble iron at simulated physiological conditions [29, 46, 47].

significantly improved iron availability (Figure 3). The same direction of results was obtained from similar test meals in humans. Davidsson *et al* [31] showed high iron absorption from the formula with pea protein and low iron absorption was previously found for the soy formula [32]. However, the iron status of the subjects differed between the two human studies, making it difficult to compare the results. Davidsson *et al* therefore recalculated the results by adjusting the ferritin level of the subjects and still found a higher absorption from the pea formula with and without phytate, compared to that of the soy formulas with and without phytate. Thus, phytate-free soy protein formulas were suggested to inhibit iron absorption more than phytate-free pea formula. The addition of ascorbic acid to the formula was also investigated, but the improvement in iron absorption by ascorbic acid could not be predicted by the gastrointestinal model because there is no differentiation between ferrous and ferric iron in the model.

Another comparison was made between dialyzability in the gastrointestinal model and absorption of non-heme iron from single meals labeled with ^{55}Fe and ^{59}Fe. The four meals consisted of fresh vegetables with white bread or

whole meal bread and corresponding lactic acid-fermented vegetables with white or whole meal bread (Sandberg *et al*, manuscript, [33]). The lactic acid fermentation was found to improve iron absorption. The fresh vegetables contained small amounts of phytate that were hydrolyzed during fermentation. The amount of iron absorbed was almost doubled when the fermented vegetables were added to a white wheat roll and also when added to the phytate-rich whole meal wheat roll. However, this iron absorption-promoting effect could not be found by *in vitro* dialyzability studies in the gastrointestinal model [20]. The model could only distinguish between the meals with white and whole meal bread, thus predicting the phytate effect (Figure 4). The insoluble complexes formed with phytate are not able to traverse the hollow fiber membranes that are used for collection of the samples from the model. The rather small molecular cut-off value of the membranes, 3–5000 Da, may be the reason for the lack of an observable difference between the meals with fresh and fermented vegetables, or it may be that the enhancing effect is related to the active uptake of iron.

It is possible to obtain high dialyzability values with small soluble complexes; e.g., citric acid and phenolic compounds, but the iron binding could still be too strong to make iron available for absorption [34, 35]. One reason for introducing the diffusability through a semi-permeable membrane was the proposition that iron bound to high molecular weight peptides may be unable to cross the mucosal mucus layer in the small intestine [4]. This may be true in some cases; e.g., for egg protein forming soluble complexes with iron but inhibiting absorption [36]. On the other hand it was recently shown that ferritin-bound iron is readily absorbed [37]. Ferritin is too large as a molecule to pass through the dialysis membrane, and the availability of iron in ferritin thus cannot be predicted by the dialyzability methods.

Very few studies have been performed correlating dialyzable zinc after *in vitro* digestion to zinc absorption.

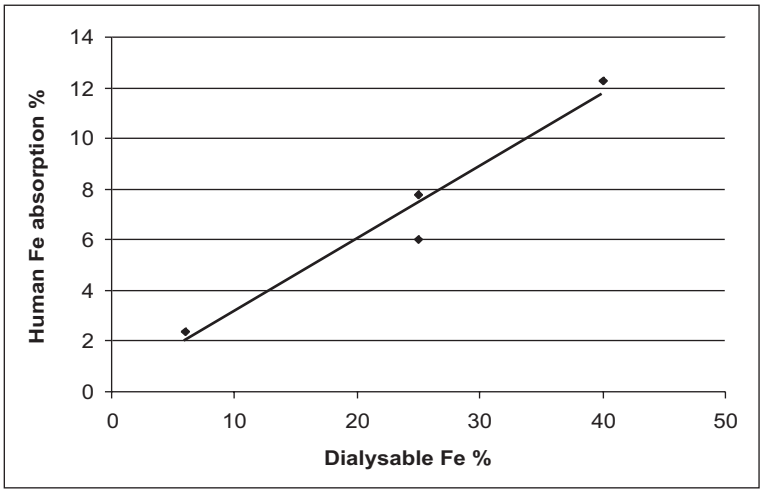

Figure 3: Comparison between human iron absorption from soy and pea protein infant formulas and the same dephytinized formulas, and dialyzable iron estimated in the TNO gastrointestinal model. Highest absorption and dialyzability was obtained from dephytinized pea protein formula (Fredriksson *et al*, manuscript) [31, 32].

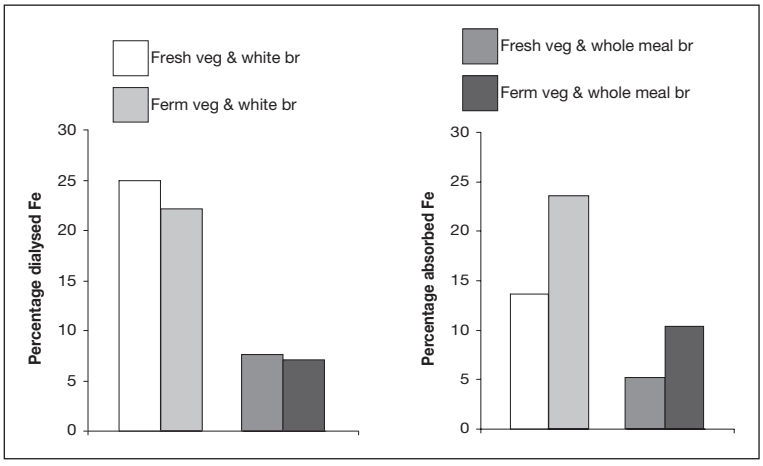

Figure 4: Percentage dialyzed iron in the TNO gastrointestinal model (left panel) and absorbed iron from single meals labeled with ^{55}Fe and ^{59}Fe in humans (right panel). The meals consisted of fresh or fermented vegetables together with white or whole meal bread (Sandberg *et al*, manuscript) [20].

Sandström and Almgren [38] measured dialyzable zinc after *in vitro* digestion of the diets, with gastric juice at pH 1 and adjustment to pH 8 and addition of trypsin, and compared the results to those from human absorption trials using single meals of the same diets. Dialyzable zinc at pH 8 was strongly correlated to *in vivo* zinc absorption from 20 composite meals labeled with ^{65}Zn.

Conclusions

In vitro solubility/dialyzability methods correlate in most cases with human studies and can therefore be used for screening purposes. Exceptions may be that effects of milk, certain proteins, tea, and certain organic acids cannot be predicted. The dialyzability methods exclude iron bound to large molecules, which in some cases is available and includes iron bound to small soluble molecules, which is not always available.

For screening purposes, simple solubility or dialyzability methods seem to be preferable to the more advanced computer-controlled dynamic gastrointestinal model. This model is likely more valuable in studies where transit time is altered or when studying different sites of digestion or dialyzability in the gastrointestinal tract. The use of a pH gradient in this model for simulating the gastric phase may be an advantage.

In vitro experiments based on solubility/dialyzability are tools to understand factors that may affect subsequent iron absorption.

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