CHAPTER III

MATERIALS AND METHODS

The study was carried out in six phases. The design of each experimental phase in terms of materials and methods is discussed separately under suitable subheads. Details of chemical analysis used for all the six phases and the statistical analysis applied on the data are given at the end of the chapter.

Phase I

As mentioned earlier, this phase dealt with a further validation of the in vitro method used in the present study since the in vivo trials showing a high correlation with this method were based on a narrow range of iron absorption values (Narasinga Rao and Prabhavati, 1978).

Twelve meals were selected from the human in vivo studies in the literature and these included diets from India (Narasinga Rao et al, 1983), North America (Hallberg et al, 1982), Latin America (Hallberg and Rossander, 1984), South East Asia (Aung-Than Batu et al, 1976) and Venezuela (Layrisse et al, 1974). These were analysed for total and in vitro available iron as well as for the enhancers and inhibitore of iron absorption present in them. The in vitro estimated iron availability values were correlated with the in vivo iron absorption values for these meals reported from the respective studies. The amount of enhancers and inhibitors as estimated in this study was compared with the food table calculated values for the same, since very few of these values were reported by the authors of the original studies.

The composition of the meals selected for this phase is given in Table 8. These meals were prepared using the same weighed quantities of the ingredients on each occasion. The recipes as outlined in the original studies were used, wherever they were available. For others, the recipes were standardised by the investigator.

Phase II

This phase dealt with the effect of five dose levels of various enhancers and inhibitors on the in vitro iron availability from a pure system, consisting of ferric chloride solution providing 3 mg elemental iron per 250 ml.

Selection of pure system

A solution of $FeCl_3.6H_2O$[1] in 250 ml of deionised water providing 3 mg elemental iron was selected as the pure system in view of the fact that studies, both in vivo and in vitro, reported in the literature by several investigators have used similar concentration of iron in the test meals (Narasinga Rao et al, 1978; Monsen and Cook, 1979; Gillooly et al, 1983; Hallberg et all, 1987; Hallberg et al, 1989). Secondly 3 mg iron per 250 ml solution represented the iron content of the standard meal homogenate used later in the study.

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1 Merck, Analytical Reagent

TABLE 8

Food compostion of the meals used in Phase I

Meal No.	Reference	Type of Meal	Ingredient	Amount (g)
1	Narasinga Rao et al, 1983	Indian; Wheat Breakfast	Wheat flour	70
			Potato	50
			Onion	25
			Oil	20
			Sugar	5
			Tea	5
			Milk	40
2	Narasinga Rao et al, 1983	Indian; Rice Lunch	Rice	200
			Redgram dal	30
			Potato	75
			Oinion	25
			Brinjal	50
			Oil	10
			Milk	160
3	Narasinga Rao et al, 1983	Indian; Ragi Breakfast	Ragi	120
			Potato	25
			Onion	20
			Brinjal	25
			Oil	20
			Sugar	5
			Tea	5
			Milk	40

Table 8 Contd . .

Meal No.	Reference	Type of Meal	Ingredient	Amount (g)
4	Narasinga Rao et al, 1983	Indian; Sorghum Breakfast	Milk	40
			Sorghum	120
			Potato	25
			Onion	20
			Brinjal	25
			Oil	2
			Sugar	5
			Tea	5
			Milk	40
			Butter	20
5	Hallberg et al 1982	Western	Hamburger; (Commercial product) containing	
			Minced meat	82
			Bread	28
			Onion sauce	75
			Brussel sprouts*	100
6	Hallberg and Rossander, 1984	Latin American	Potatoes	200
			Maize	80
			Black beans	31
			Rice	50

* Due to unavailability, it was replaced with cabbage (100 g).

Meal No.	Reference	Type of Meal	Ingredient	Amount (g)
7	Aung-Than Batu et al, 1976	South East Asian; Rice meal	Rice	200
			Spinach	40
			Fish paste	8
			Beans	25
			Onion	2
			Oil	3
			Salt	0.9
8	Aung-Than Batu et al, 1976	South East Asian; Rice-fish meal	Rice	200
			Catfish	40
			Spinach	40
			Fish paste	8
			Beans	25
			Onion	4
			Garlic	0.33
			Chilli	1.11
			Ginger	2.22
			Salt	1.99
			Groundnut oil	13.15
9	Layrisse et al, 1974	Venezuelan ; Central Andes area, Breakfast	Maize	40
			Butter	10
			Cheese	15
			Oats	20
			Milk	200

Table 8 Contd . .

Meal No.	Reference	Type of Meal	Ingredient	Amount (g)
10	Layrisse et al, 1974	Venezuelan ; Central Andes area, Lunch	Maize	40
			Black beans	10
			Fish paste	7
			Plantain	80
			Tomato	50
			Potato	40
			Carrot	40
			Meat	50
11	Layrisse et al, 1974	Venezuelan ; Coast area, Lunch	Maize	40
			Rice	10
			Plantain	90
			Fish	50
			Avocado*	50
			Tomato	50
			Onion	10
			Watermelon	150
12	Layrisse et al, 1974	Venezuelan ; Coast area Supper	Maize	40
			Milk	100
			Plantain	90
			Fish	50
			Pumpkin	100
			Papaya	100
			Coffee	8
			Sugar	8

* Due to unavailability, it was omitted from the meal

Selection of levels of enhancers and inhibitors

The enhancers that were tested in the present study were ascorbic acid and citric acid. The inhibitors tested were tannate, phytate, oxalate and calcium-phosphate. These were selected in view of the fact that they represented the commonest dietary factors affecting iron availability from Indian vegetarian meals. Meat, fish and poultry were not included as they are consumed rarely and in negligible amounts by a majority of the population.

Since the content of these dietary constituents vary widely in commonly consumed Indian meals, it was necessary to select a range of these enhancers and inhibitors that represented the average daily intake of these food constituents. The following sets of data were utilized in arriving at the range of these enhancers and inhibitors :

(a) A diet survey conducted by the investigator on low, middle and high income group women (n = 6 in each group) purposively selected from vegetarian families of urban Baroda. The details of the diet survey are discussed in Phase IV under 'selection of standard meal'.

(b) Recommended Dietary Allowances for an adult man (sedentary and moderate worker) by the Indian Council of Medical Research (ICMR, 1989a).

(c) Chemical analysis of diet for a reference adult man (moderate worker) as recommended by ICMR (Puar, 1983).

(d) Dietary data from National Nutrition Monitoring Bureau (NNMB, 1981) for the rural population of India from three different income groups.

(e) Dietary data from NNMB (1984) for the urban population of India from three different income groups

(f) Dietary data from Diet Atlas of India (1969) regarding the avilability of food per person per day.

From each of the above sources, calculations were made for dietary content of total iron, ascorbic acid, citric acid, tannate, phytate, oxalate, calcium and phosphorus, using the food tables (Gopalan et al, 1989; Diem and Lentner, 1970) and other sources of published data on the content of these dietary constituents in foods (Rao and Deosthale, 1982; Narasinga Rao and Prabhavati, 1982; Gillooly et al, 1983; Rao and Deosthale, 1988).

A wide range was arrived at for each of the six food constituents as shown in Table 9. Since the iron content of the pure system was 3 mg, the ranges were recalculated for 3 mg dietary iron intake. These are shown in Table 10. Since citrate content in a day's diet is contributed mainly by the fruits consumed, only once in a day, and is not divided over 3-4 meals in a day, the entire range for daily intake of citrate was taken for selection of the dose levels.

TABLE 9

Range of dietary intake (per caput unit per day) of iron and various enhancers and inhibitors present in Indian meals

Dietary constituent	Range of dietary intake per caput unit/day (mg)
Total Iron	13 to 54
Ascorbic Acid	17 to 146
Citric Acid	95 to 450
Tannate	398 to 1589
Phytate	462 to 3846
Oxalate	43 to 862
Calcium	271 to 1266
Phosphorus	800 to 2783

TABLE 10

Range of dietary intake of enhancers and inhibitors for 3 mg iron intake

Dietary constituent	Range of dietary intake for 3 mg iron (mg)
Ascorbic Acid	3 to 16
Citric Acid	95 to 450
Tannate	25 to 350
Phytate	58 to 750
Oxalate	5 to 52
Calcium	21 to 240
Phosphorus	83 to 440

Using the above ranges, five dose levels of each of the six dietary constitutents were selected in such a way that the entire range for daily intake of these food constituents in Indian meals was represented. The selected dose levels are shown in Table 11. The selected levels of these enhancers and inhibitors were dissolved in double distilled water and added separately to the pure system[1] as shown below, to study the dose effect on iron availability :

Source of Iron (SI)
(3 mg Fe/250 ml)

+

Enhancer 1 to n
(L1 to L5)

OR

Inhibitor 1 to n
(L1 to L5)

Phase III

In this phase, the interaction effect of enhancers and inhibitors, added at two selected levels in various combinations of each other was investigated in the pure system. On the basis of the dose response curves obtained in Phase II, two levels of each of the enhancers and inhibitors were selected for the interaction effect, as shown in Table 12. All possible combinations were generated through an IBM-compatible PC assembly, using the two selected levels of the six variables (2^6 = 64). The interaction effect was studied by adding the six

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1. The volume was made upto 250 ml after addition of enhancers and inhibitors.

TABLE 11

Selected dose levels of various enhancers and inhibitors per 3 mg iron

Variable	Dose Levels (mg)				
	L_1	L_2	L_3	L_4	L_5
Ascorbic Acid	5.6	9.3	23.2	46.5	186
Citric Acid	30	240	480	720	960
Tannate	25	100	200	400	600
Phytate	50	250	500	750	1000
Oxalate	5	25	50	75	100
Calcium and Phosphate *	20:40	40:80	80:160	160:320	320:640

* Calcium and phosphate were added together in the ratio of 1:2 since it has been shown that when they are present together, they reduce iron absorption significantly (Monsen and Cook, 1976).

TABLE 12

Levels of enhancers and inhibitors selected for studying the interaction effect in the pure system

Variable	Levels (mg) L_1	L_2
Ascorbic Acid	5.6	46.5
Citric Acid	30	960
Tannate	100	600
Phytate	50	1000
Oxalate	5	100
Calcium and Phosphate	20: 40	320: 640

dietary constituents together, in 64 combinations to the pure system, as shown below:

SI
+
Enhancer 1 to n
(L 1 and L 2)
+
Inhibitor 1 to n
(L1 and L 2)

In 64 Combinations

Based on the data obtained, a regression equation was formulated for predicting the in vitro availability of iron. This was termed as equation 1.

Phase IV

In this phase, dose effect of different enhancers and inhibitors was studied on availability of iron from a standard cereal meal.

Selection of the standard meal

For the experiments of this phase and the next phase, a cereal meal, designated as standard meal, was formulated based on the results of a diet survey, which was carried out to obtain information regarding the general meal pattern, most commonly consumed cereal meal and the average daily intake of food by adult women in Gujarat (India).

Subjects

The subjects for the diet survey consisted of 18 women in the age group of 22 y to 55 y. They were purposively selected from vegetarian, Gujarati families of urban Baroda. These women were categorised into three categories of six women each, based on their per capita income and education level as shown below:

Income Group	Per Capita Income per month	Education Level
High Income Group (HIG)	Above Rs.1200	Graduate or above
Middle Income Group (MIG)	Rs.400 to Rs.750	XII Standard or Graduate
Low Income Group (LIG)	Below Rs.150	Middle School or Illiterate

Tool for collection of dietary information

Twenty four hour recall method was used for collecting information regarding the general meal pattern, most commonly consumed cereals and the average daily intake of food by women.

Since the menu of the recall day may be atypical and may not be representative of what is eaten over a week, it was decided to record the dietary information for three consecutive days. The amount of cooked foods and raw ingredients using the standard measures was recorded in a proforma developed and pretested by the invetigator.

Formulation of standard meal

From the dietary information collected, the average intake in terms of various food groups was calculated using the food tables (Gopalan et al, 1989). This is shown in Table 13. It can be seen from the Table that there was a wide variation in the quantity of food consumed by the women belonging to the three income groups. However, there was no appreciable difference in the type of cereals, pulses and vegetables consumed by the women of the three income groups.

In order to select the main meal of the day with respect to its iron content, calculations were made for percentage contribution through various meals, to the total daily iron intake in the three income group women. This is indicated in Table 14.

It can be observed from the Table that the mean total daily iron intake was 20.7 mg, the lowest being in LIG and the highest in HIG women. However, in all the three groups, the maximum % contribution to the total iron intake was through the lunch meal (60%) followed by dinner (35%). Breakfast and evening snack contributed little to the total intake of iron (4% and 2% respectively). In view of these observations, the most commonly consumed food items in amounts equivalent to the mean dietary intake through lunch by the women were selected for formulation of the standard meal. The four food items selected for formulation of the standard meal in the present study were also

TABLE 13

Mean daily intake of food by the three income group women

(n=18)

Food Group	HIG	MIG	LIG	Mean ±SD
Cereals (g)	110	159	146	139 ± 44
Pulses (g)	45	46	29	40 ± 15
Vegetables and Fruits (g)	259	223	129	204 ±142
Milk and Milk products (ml)	321	229	79	210 ±151

TABLE 14

Percentage contribution by different meals to the total daily intake of iron in women belonging to the three income groups

Income Group	Total daily iron intake (mg)	% Contribution by meals			
		Breakfast	Lunch	Snacks	Dinner
HIG	24.3	3	61	6	30
MIG	20.7	7	54	0.4	39
LIG	17.1	0.8	63	0.4	35
Mean	20.7	3.6	60	2.2	35

shown to be the most commonly consumed food items in another diet survey on one hundred women in urban slums of Baroda (Seshadri, 1987). Compostion of the standard meal is shown in Table 15.

The recipe preparation of the meal was recorded through observation in the homes and it was formulated in the same manner in the laboratory using standardized methods and weighed quantities of the food ingredients everytime for analysis. Detailed method of preparation of the standard meal is given in Appendix I. Prior to any experiments, the standard meal was chemically analysed for iron content, content of enhancers and inhibitors and proximate principles (Table 15).

Addition of enhancers and inhibitors

The five dose levels, similar to those indicated in Phase II were used for studying the dose relationship of different enhancers and inhibitors to in vitro iron availability from the standard meal. These were added to 250 ml of the meal homogenate providing 3 mg nonheme iron. Processing of the meal is described in detail later in this capter. Addition of enhancers and inhibitors was carried out as shown below, after taking into consideration the quantity of these, endogenously present in the standard meal:

TABLE 15

Composition of the standard meal

Item	Ingredient	Amount (g)
	Food Composition	
Rotla[1]	Wheat flour (Triticum aestivum)	50
Khichdi[2]	Rice (Oryza sativa)	20
	Redgram dal (Cajanus cajan)	10
Kadhi[3]	Bengalgram flour (Cicer arietinum)	5
	Curds	20
	Oil	2.5
Vegetable[4]	Potatoes (Solanum tuberosum)	80
	Onions (Allium cepa)	20
	Oil	5
	Salt and Spices	3
	Nutrient Composition[5] (dry weight basis)	
Energy (Kcal)		366
Fat (g)		7.65 ± 0.11
Protein (g)		14 ± 0.31
Carbohydrate (g)		60
Fibre (g)		0.42 ± 0.21
Ash (g)		2.55 ± 0.22
Iron (mg)		7 ± 0.07
Ascorbic acid (mg)		-
Citric acid (mg)		-
Tannic acid (mg)		-
Phytic acid (mg)		5.5 ± 0.11
Oxalic acid (mg)		22 ± 1.11
Calcium (mg)		10 ± 0.59
Phosphorus (mg)		118 ± 2.15

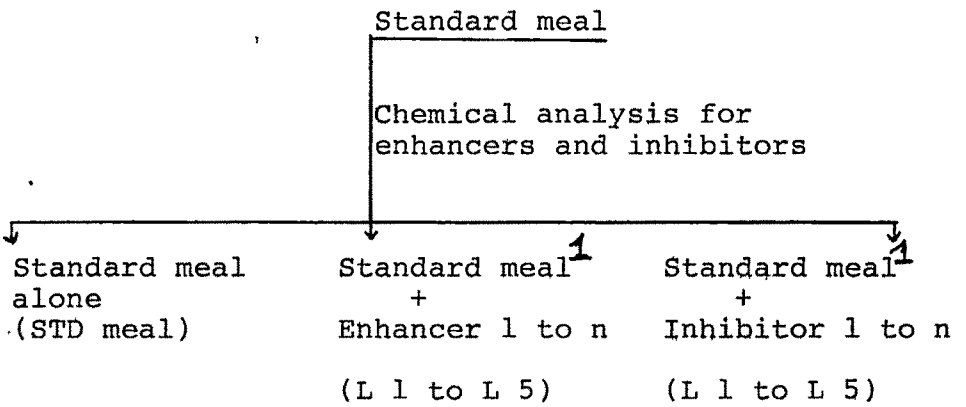
1 Rotla is a thick wheat chapati.

2 Khichdi - rice and dal cooked together for 30 minutes till soft.

3 Kadhi - Curry prepared from buttermilk and Bengalgram flour brought to boil (1 minute).

4 Vegetable - potatoes and onions chopped and shallow fried for 7 minutes, till tender.

5 Each value indicates mean of four replicates.



A sub-study under Phase IV

Data obtained from the preceeding three phase namely Phase II, III and IV revealed that two dietary constitutents viz. phytate and oxalate, out of the six factors studied, showed results that were opposed to what has been reported in human in vivo studies. Both these constitutents increased rather than decreased the in vitro availability of iron from the pure system as well as from the standard meal.

In order to explain the unexpected trends obtained with the pure forms of phytate some experiments were carried out to study its effect in a bound form, as bran or as a complex of mono-ferric phytate and Mg-K phytate.

Trials with bran

The effect of bran or dephytinised bran on iron availability was studied from the pure system, containing a total of 3 mg iron

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1. The enhancers and inhibitors were added to an aliquot of STD meal homogenate providing 3 mg iron; these were then rehomogenised and volume was made upto 250 ml.

per 250 ml of the solution. Bran was purchased locally[1]. For dephytinising the bran, 0.5N HCl was used, as described by Hallberg et al (1987). The follwoing experiments were carried out :

(a) Wheat bran added in amounts equivalent to 50, 100, 150 and 200 mg phytate.

(b) Rice bran added in amounts equivalent to 50, 250, 500 and 750 mg phytate.

(c) Dephytinised wheat bran added in amounts equivalent to those in experiment (a).

(d) Dephytinised rice bran, added in amounts equivalent to those in experiment (c).

Trials with mono-ferric —phytate and Mg-K-phytate

Effect of a complex of mono-ferric-phytate and Mg-K-phytate (in a ratio of 1:3) providing 50, 100, 150 and 200 mg phytate, on iron availability was studied from the pure system. Mono-ferric phytate was prepared from Na-phytate and $FeCl_3.6H_2O$, as described by Morris and Ellis (1976).

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1 Kohinoor Mills, Baroda for wheat bran and Agarwal Rice Mills, Bajuwa for rice bran.

Phase V

This phase dealt with the interaction effect of selected enhancers and inhibitors on iron availability from the standard meal. Since the major goal of the present phase was to evolve a predictive model based on the dietary consitutents that showed expected trends with iron availability, as those observed in human, in vivo studies, phytate and oxalate were not included for the experiements of this phase, in view of the results obtained in the sub study of Phase IV. These are discussed later in the thesis. The remaining four variables namely ascorbic acid, citric acid, tannic acid and calcium and phosphate were studied for their interaction effect.

The two selected dose levels, used in this phase were similar to the ones used in the pure system (Phase III) for all the variables except ascorbic acid. Preliminary trials with three levels of ascorbic acid viz 5.6 mg, 46.5 mg and 186 mg revealed that the first two levels were insufficient to overcome the interaction effect of added inhibitors in the standard meal. Therefore it was decided to use 46.5 mg and 186 mg as the low and the high level of ascorbic acid respectively for studying the interaction effect from the standard meal. The two selected dose levels for each of the enhancers and inhibitors used in this phase, are shown in Table 16.

The enhancers and inhibitors were added in various combinations ($2^4 = 16$) to the standard meal, as shown below :

TABLE 16

Levels of enhancers and inhibitors selected for studying the interaction effect from the standard meal

Variable	Levels (mg)	
	L_1	L_2
Ascorbic Acid	46.5	186
Citric Acid	30	960
Tannate	100	600
Calcium and Phosphate	20: 40	320: 640

STD Meal
+
Enhancer (1 to n)
(L 1 and L 2)
+
Inhibitor (1 to n)
(L 1 and L 2)

} In 16 Combinations

Based on the data obtained, a regression equation was evolved which was designated as equation 2.

Phase VI

In this phase, an attempt was made to evaluate the predictive powers of the two equations evolved from the pure system (equation 1) and the standard meal (equation 2) respectively, as applied to ten, typical Indian meals.

Selection of typical Indian meals

For this, results of diet surveys carried out in four major regions of India namely North (Delhi), West (Gujarat), East (Assam) and South (Tamil Nadu) by other investigators in our Department (Billa, 1990) and by Narasinga Rao et al (1983) were utilised. The recipe and food composition of the selected 10 meals is given in Table 17. The meals were prepared using standardised methods of cooking and weighed quantities of the ingredients as reported by the above investigators. Details of meal preparation are given in Appendix II. Four replicates were analysed for each meal for the in vitro iron availability,

TABLE 17

Food compostion of the typical Indian meals used in Phase VI

Meal No.	Reference	Item	Ingredient	Amount (g)
1	Narasinga Rao et al, 1983	Paratha	Wheat flour	70
			Oil	15
		Vegetable	Potato	50
			Onion	15
			Oil	5
			Lemon juice	25(added)
		Tea	Tea	5
			Sugar	20
			Milk	40
2	Narasinga Rao et al, 1983	Rice	Rice	200
		Dal	Redgram dal	30
			Oil	2.5
		Vegetable	Potato	75
			Oinion	25
			Brinjal	50
			Oil	7.5
			Lemon juice	25(added)
		Milk	Milk	160
3	Narasinga Rao et al, 1983	Mudde	Ragi	120
			Oil	18
		Vegetable	Potato	25
			Onion	20
			Brinjal	25
			Oil	2

Table 17 Contd . .

Meal No.	Reference	Item	Ingredient	Amount (g)
3	Narasinga Rao et al, 1983	Tea	Tea	5
			Sugar	5
			Milk	40
4	Narasinga Rao et al, 1983	Rotla	Sorghum	120
		Butter	Butter	20
		Vegetable	Potato	25
			Onion	20
		Tea	Tea	5
			Sugar	5
			Milk	40
5	Billa, 1990	Rice	Rice	100
		Sambhar	Redgram dal	12
			Tamarind	3
			Brinjal	20
			Curry leaves	1
			Mustard seeds	0.5
			Spices and salt	3
			Oil	2.5
		Rasam	Redgram dal	12
			Tamarind	13
			Tomatoes	40
			Mustard seeds	0.5
			Spices and Salt	4
			Oil	2.5

Table 17 Contd . .

Meal No.	Reference	Item	Ingredient	Amount (g)
5	Billa, 1990	Vegetable	Cabbage	100
			Mustard seeds	0.5
			Spices and Salt	2
			Oil	2.5
6	Billa, 1990	Rice	Rice	100
		Sambhar	(Same as in Meal No.5)	
7	Billa, 1990	Stuffed Parathas	Wheat flour	60
			Potatoes	80
			Spices and Salt	3
			Oil	5
		Curds	Curds	100
8	Billa, 1990	Chapati	Wheat flour	75
		Vegetable	Brinjal	30
			Potatoes	25
			Onion	10
			Spices and Salt	3.5
			Oil	2.5
		Tea	Tea	5
			Sugar	10
			Milk	50

Table 17 Contd . .

Meal No.	Reference	Item	Ingredient	Amount (g)
9	Billa, 1990	Chapati	Wheat flour	75
		Vegetable	Cauliflower	40
			Potatoes	20
			Spices and Salt	3.5
			Oil	2.5
		Dal	Greengram dal	20
			Spices and Salt	3.5
			Oil	1.5
		Curds	Curds	50
10	Billa, 1990	Rice	Rice	100
		Dal with Vegetables	Lentil dal	20
			Bottlegourd	30
			Potatoes	25
			Onion	10
			Spices and Salt	2.5
			Oil	2.5

ascorbic acid, tannate, phytate, oxalate, calcium and phosphorus. Citrate values were taken from the scientific tables (Diem and Lentner, 1970).

The two prediction equations evolved from the pure system (equation 1) and the standard meal (equation 2) respectively were applied to the ten Indian meals for predicting the in vitro availability of iron, based on the amount of enhancers and inhibitors present in them. The calculated values for iron availability were correlated with the actual estimated values to evaluate the predictive powers of the equations.

Chemical Analysis

The parameters analysed in various phases of the study are shown in Table 18.

Processing of the meals

Homogenisation

The standard meal as well as the meals used in Phase I and Phase VI were homogenised to a creamy consistency with minimum amount of double distilled water in Sumeet Mixer[1]. Total volume of the homogenate was recorded and suitable aliquots were taken for analysis of total iron, soluble and ionisable iron and ascorbic acid.

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1 Sumeet Machines, Bombay, India (Stainless steel assembly).

TABLE 18

Parameters analysed in various experimental phases

Parameters	Phase I	Phase II	Phase III	Phase IV	Phase V	Phase VI
1. Total Iron	✓	✓	✓	✓	✓	✓
2. In Vitro Available Iron (Soluble and Ionisable Iron)	✓	✓	✓	✓	✓	✓
3. Ascorbic Acid	✓	-	-	✓	-	✓
4. Tannic Acid	✓	-	-	✓	-	✓
5. Phytin-Phosphorus	✓	-	-	✓	-	✓
6. Oxalate	✓	-	-	✓	-	✓
7. Calcium	✓	-	-	✓	-	✓
8. Phosphorus	✓	-	-	✓	-	✓
9. Moisture	✓	-	-	✓	-	✓
10. Protein	✓	-	-	✓	-	✓
11. Fat	✓	-	-	✓	-	✓
12. Fibre	-	-	-	✓	-	-
13. Ash	-	-	-	✓	-	-

Drying

For analysis of proximate principles, phytin-phosphorus, oxalate, tannate, calcium and phosphorus, dry powder of the diet samples was used. The meal homogenates were dried in an air oven at 55-60° C till two consecutive weights were identical (AOAC, 1984). The dry diets were powdered in the glass jar of Sumeet grinder till all of it passed through a 0.1 mm sieve.

These diet powders were packed in autoseal polythene bags and stored in glass jars with non-metal double lids, in refrigerator. Suitable portions were taken, for analysis of proximate principles, phytin-phosphorus, oxalate, tannate, calcium and phosphorus.

Estimations

All estimations whether on fresh diet samples or on dry diet powders, were carried out on four replicate samples. Readings were taken in duplicate for each of the four replicates unless otherwise mentioned. The details of the parameters and the methods used for each, are given below.

Total Iron

Initial comparisons were made between wet ashing vs dry ashing of the diet samples for analysis of total iron. It was found that there was no significant difference between the values obtained by the two methods ('t' nonsignificant at $p < 0.001$). In all subsequent analysis, wet ashing was used for digesting the diet samples. Total iron was estimated in the digestate using potassium thiocyanate as described by Wong (Oser, 1980).

Principle : Iron is released from the food by digestion with acid mixture and then it is treated with thiocyanate which forms a red coloured complex with iron that can be colorimetrically measured. Though this reagent is not as sensitive as the dipyridyl, bathophenanthroline phosphate or ferrozine reagent, it can measure both Fe++ and Fe+++ forms of iron while the other three reagents are specific only for Fe++ form of iron (Reddy et al, 1988; Chidambaram et al, 1989). Since the cereal meals used for the present study contained both ferrous and ferric forms of iron in appreciable quantities, it was considered appropriate to use the thiocyanate method for estimating the total iron content of the meals. This method has been shown to give comparable results for total iron content of foods using dipyridyl for colour development (Narasinga Rao and Prabhavati, 1978).

Wet Digestion : Prior to any chemical analysis, the food samples were digested for liberation of iron in the digestate. For this, five ml of the diet homogenate was measured accurately and transferred to a Kjeldahl's digestion flask. To this, 25-30 ml of a mixture of concentrated nitric acid and sulfuric acid (5:1, v/v) was added. The mixture was swirled lightly and heated in a digestion chamber for 3-5 hrs, until a colourless, clear solution was obtained. The flask was then allowed to cool and the contents poured into a 100 ml volumetric flask. The digestion flask was washed 2-3 times with double distilled water and the washings were added to the volumetric flask to ensure

complete removal of iron in the solution, after which the volume was made up to the mark. From this, suitable aliquots were taken for the estimation of total iron.

Estimation : Iron was estimated in the digestate by the method of Wong (Oser, 1980). Ten ml of the sample solution containing iron as well as a blank of 10 ml double distilled water was taken in test tubes. To each, 0.5 ml of saturated potassium persulfate solution was added, followed by 2 ml of 3 N potassium thiocyanate solution. The tubes were mixed by inversion and read within the next 30 min in the Spectrophotometer, setting the instrument to zero with the blank at 480 nm. Aliquots of standard iron solutions prepared from crystalline ferrous ammonium sulfate, containing 10-50 µg ferric iron were also run simultaneously through the entire procedure. The readings of the unknown were compared with those of the standard solutions in order to obtain the concerntration of iron in the unknown solution.

The amount of iron present in the food sample was calculated as follows :

$$\text{Iron content of the meal (mg)} = \frac{\text{Reading of the sample}}{\text{Reading of standard}} \times \text{Concentration of standard } (\mu g) \times \frac{100}{10} \times \frac{\text{Volume of Homogenate}}{5} \times \frac{1}{1000}$$

In vitro availability of iron

This was estimated by the method proposed by Narasinga Rao and Prabhavati (1978).

Principle : This method is based on extraction of iron from foods with pepsin-HCl at pH 1.35, followed by changing the pH of the soluble extract to 7.5 in order to simulate the conditions in stomach and duodenum respectively. Iron which is determined in the final filterate with dipyridyl , represents the availability of iron in vitro.

Liberation of iron from the food samples : For this, twenty five ml aliquots of the diet homogenate were taken in 150 ml conical flasks (with glass stoppers) and the pH was recorded. To this, 25 ml of pepsin-HCl mixture (pepsin from Sigma, 1:10,000) was added. The pH of the solution was adjusted to 1.35 by dropwise addition of 6N HCl. The mixture was incubated for exactly 90 min at 37 ^{o}C in a mechanical shaker (100-120 oscillations/min). The contents of the flask were then centrifuged at 3000 rpm for 30 min and the supernatant was transferred to a clean 150 ml conical flask. The weight of the supernatant was recorded and it was heated in a boiling water bath for 15 min, cooled and reweighed. The loss in weight due to evaportion was made up with double distilled water. The volume of the solution was then recorded after which it was filtered through Whatman no. 44 filter paper. The pH of the filterate was adjusted to 7.5 using NaOH solutions of varying strengths (0.5N to 0.1 N) taking care to record the total amount of NaOH added.

The mixture was then incubated in the shaker-water bath for 45 min at 37°C after which it was filtered using Whatman no. 44. The filterate was used for the estimation of soluble iron and ionizable iron.

Soluble Iron : Soluble iron was estimated by the method of Tennant and Greenman (1969).

(a) Principle : Iron was released from the food iron complexes, in solution, by digestion with acid-permanganate solution at room temperature. Excess permanganate was reduced with ascorbic acid and iron in the resulting clear solution was estimated using dipyridyl as described under ionizable iron. This method determines the iron in many soluble biological complexes. Therefore, the fraction of iron, estimated using the above method is termed as soluble iron.

(b) Estimation : Six ml of the final filterate was taken in a test tube to which 4 ml of acidified KMnO4 was added and the mixture was shaken and left at room temperature for 15 min. Two ml of 20% ascorbic acid was added to this and the mixture was incubated for 2 hours at 56°C in an ordinary water bath.

The solution was filtered through Whatman no.44 and iron in the filterate was estimated using dipyridyl as described in the next section on ionizable iron. Soluble iron was calculated by comparing the readings of samples with those of standards run

simultaneously. Sample blanks were run in order to cancel the effect of colour due to extraneous material.

$$\text{Soluble iron in the meal (mg)} = \frac{\text{Reading of sample}}{\text{Reading of standard}} \times \text{Conc. of standard } (\mu g) \times \frac{12}{7} \times \frac{50+\text{Volume of NaOH}}{6} \times \frac{\text{Volume of Homogenate}}{25} \times \frac{1}{1000}$$

Ionizable Iron : Ionizable iron was estimated in the final filterate (after extraction of food sample in solution by pepsin-HCl) by the dipyridyl method.

(a) **Principle :** Dipyridlyl forms a pink colour with ionized forms of iron at pH 4.2 (of the acetate buffer), the intensity of which can be read colorimetrically at 540 nm. Since this method is specific for only the ionised form of iron, the fraction, estimated by this method is termed as ionizable iron.

(b) **Estimation :** For estimation of ionizable iron, aliquots of 7 ml of the final filterate were taken in test tubes to which 1 ml of 10% hydroxyalamine hydrochloride, 5 ml of acetate buffer (pH 4.2) and 2 ml of α,α dipyridyl solution were added in that order. The color intensity was read after 30 min in the Spectrophotometer, after adjusting it to zero with the reagent blank, at 540 nm. Sample blanks were also prepared in the same manner and their readings were subtracted from the sample readings to cancel the effect of

colour due to presence of extraneous material in the filterate.

The corrected readings of the unknown solutions were compared with those of standard solutions containing 2 to 10 µg iron ($FeSO_4.7H_2O$) which were run in the same manner as the samples.

$$\text{Ionizable Iron in the meal (mg)} = \frac{\text{Corrected Reading of the sample}}{\text{Reading of Standard}} \times \text{Conc. of Standard (µg)} \times \frac{50+\text{Volume of NaOH}}{7} \times \frac{\text{Volume of Homogenate}}{25} \times \frac{1}{1000}$$

The ionizable iron was expressed as a percentage of the total iron as follows.

$$\% \text{ Ionizable Iron} = \frac{\text{mg Ionizable Iron}}{\text{mg Total Iron}} \times 100$$

% In vitro available iron : It was calculated on the basis of the % ionizable iron using the prediction equation of Narasinga Rao and Prabhavati (1978), as shown below :

$$Y = 0.4827 + 0.4707\ X$$

where X = % ionizable iron

Y = % in vitro available iron

Ascorbic acid

Ascorbic acid was extracted from fresh diet homogenates by metaphosphoric acid-acetic acid mixture and analysed by the titrimetric method using 2,6, dichlorophenol indophenol dye (AOAC, 1984).

Principle : In this method, the vitamin is extaracted and titrated in the presence of metaphosphoric acid-acetic acid and/or sulfuric acid mixture to maintain proper acidity for reducing reaction of ascorbic acid on the dye and to avoid auto-oxidation of the vitamin at high pH.

Preparation of sample assay solution: Twenty five ml aliquots of fresh diet homogenates were taken in conical flasks and tested for presence or absence of basic substances by placing a few drops of thymol blue pH indicator on the homogenate in a spot plate. A yellow colour or pH> 1.2 indicated appreciable amounts of basic substances. In such cases, the pH was adjusted to 1.2 (or a red colour on testing with thymol blue) with metaphosphoric acid - acetic acid - sulfuric acid mixture. Volume was then made upto 50 ml with metaphosphoric acid - acetic acid solution and centrifuged. The supernatant was transferred to another clean conical flask and triplicate aliquots of 7 ml each were taken for titration.

Titration: Aliquots of 7 ml each were transferred to 50 ml conical flasks. Rapid titration was performed with indophenol dye from a 50 ml burette until light but distinct rose pink colour persisted for >5 sec. Standard solutions containing 1-5 mg ascorbic acid were also titrated by taking triplicate aliquots of

2 ml each in 50 ml conical flasks containing 5 ml of metaphosphoric acid - acetic acid solution. Similarly three blank solutions, consisting of 7 ml metaphosphoric acid - acetic acid solution plus volume of double distilled water equal to volume of indophenol solution used in direct titrations were also titrated. After subtracting average blank reading (usually 0.1 ml) from standard titrations, calculations were made to express concentration of indophenol solution as mg ascorbic acid equivalent to 1 ml reagent. Indophenol solution was standardized daily with freshly prepared ascorbic acid standard solution.

Correction for the presence of other reducing substances : Since certain other reducing substances such as ferrous (Fe^{+2}), stannous (Sn^{+2}) and cuprous (Cu^{+2}) ions interfere with this method, corrections had to be made for the presence of these ions. For this, simple tests were carried out to determine whether these reducing ions were present in such amounts so as to interfere with the results or not.

(a) **Test for the presence of Cu^{+2} and Fe^{+2} :** Two drops of 0.05% acquous solution of methylene blue was added to 10 ml freshly prepared solution (1:1) of sample solution and metaphosphoric acid - acetic acid solution and mixed. Disappearance of blue colour in 5-10 sec indicated presence of interfering substances such as ferrous, cuprous and other ions.

(b) **Test for the presence of Sn^{+2} :** For stannous, another 10 ml of sample solution to which 10 ml HCI (1+3) had been added, was taken and 5 drops of 0.05% acquous solution of indigo carmine was added and mixed. Disappearance of colour in 5-10 sec indicated the presence of Sn^{+2}.

These tests were carried out on all sample solutions and corrections were made colorimetrically, wherever required. Ascorbic acid content of the meals was calculated as follows

$$\text{Ascorbic acid in the meal (mg)} = \frac{\text{Corrected reading (Titre - Blank)}}{\text{ml equivalent of the dye for 1 mg ascorbic acid}} \times \frac{50}{7} \times \frac{\text{Volume of homogenate}}{25}$$

Tannic acid

Tannic acid in the dry diet powders was extracted with methanol and assayed using the modified vanillin reaction method (Price et al, 1978). Since catechin was used as the standard, the values were expressed as catechin equivalents.

Principle : Tannic acid forms a pink coloured complex with vanillin in acid medium, which can be estimated colorimetrically.

There are a number of published methods available for analysing tannins (Wisdom et al, 1987; Armory and Schubert, 1987; Makkar et al, 1987; Inove and Hagerman, 1988; Brune et al, 1989).

These methods can be categorised into two groups -- chemical assays and protein binding assays.

Chemical assays that are used for determining the amount of tannin in a sample are more appropriate for our purpose than protein-binding assays which are more useful for determining the potential biological activity of tannins in a sample.

Under chemical assays for estimating tannins, the vanillin assay (Price et al, 1978) has been indicated to be adequately sensitive for most applications. The other methods like the prussian blue assay or Folin-denis assay do not discriminate between phenolics and other easily oxidised materials such as ascorbic acid. Therefore, for the purpose of estimating tannins from cereal meals in the present study, the modified vanillin reaction assay was selected (Price et al, 1978).

Extraction of tannins in solution : Triplicates of 1 g dry diet powders were weighed accurately and were extracted in 10 ml of distilled methanol in capped, rotating test tubes for 20 min. They were centrifuged for 10 min at 5000 rpm and the supernatant was used for the assay at 30 ^{o}C.

Estimation : All the reagents as well as the sample supernatants were kept at 30 ^{o}C in a water bath, prior to the estimation. Vanillin reagent was prepared freshly by mixing equal volumes of 1% vanillin in distilled methanol and 8% conc HCl in methanol. From the sample supernatant, four replicate aliquots of one ml each were taken in test tubes and 5 ml of the vanillin HCl reagent was added to the tubes at 1 min interval, at

30 °C. For sample blank, another 1 ml aliquot of the sample solution was taken in a test tube and 5 ml of 4% conc HCl in distilled methanol was added at 1 min. interval. Reagent blank consisted of 1 ml of distilled methanol and 5 ml of vanillin-HCl reagent. All the test tubes were kept in a water bath at 30 °C for 20 min after which, they were removed one by one and the absorbance at 500 nm read in a Spectrophotometer.

A standard curve was constructed using catechin (Sigma Chemicals) standard upto 1 mg cncentration, treating it in the same manner as the samples. Tannin content of the meal was calculated as follows :

$$\text{Tannins expressed as catechin equivalents in the meal (mg)} = \frac{\text{Corrected Reading of Sample}}{\text{Reading of the Standard}} \times \text{Conc. of Standard (mg)} \times 10 \times \frac{\text{Dry weight of the meal}}{1}$$

Phytate

Phytate was estimated as phytin-phosphorus in the meals by the method of AOAC (1984).

Principle : In this method phytate is extracted from the sample by treatment with 0.5 N HCl, precipitated as ferric phytate which is treated with NaOH to precipitate ferrichydroxide, and bring Sodium phytate into solution, which is digested with sulfuric and per chloric acid to destroy all organic material and briny phytin - phosphorus into solution.

Phosphorus in the final digestate is then measured by the method of Fiske and Subbarow (Oser, 1980).

Quantitative estimation of phytate may be based on the anlysis of phytin-phosphorus that is bound to the myo-inositol-moiety or estimation of iron in the isolated ferric phytate precipitate. Indirectly, the estimation can also be done by determining the amount of residual iron left in the solution after precipitation of ferric phytate from a known concentration of ferric salt in acid solution (Makower, 1970).

Though the method recommended by Makower (1970) where iron remaining in solution is measured after initial conversion to ferric hydroxide, has the advantage of improved separation from interfering substances over the determination of iron or phosphorus in ashed ferric phytate, this method requires the use of equipment such as refrigerated ultracentrifuge. Also it is not feasible for estimating phytate in large number of diet samples. Therefore, the method recommended by AOAC (1984) where phytate is determinded as phytin-phosphorus in the ashed solution was selected for the present study due to its greater speed and convenience.

Extration : Five grams of the dried food sample was shaken in a 150 ml conical flask with 100 ml of 0.5 N HCl for 2 hrs. The extract was filtered and the first few ml of the filterate were discarted. Twenty ml of the filterate was taken and it was neutralised with 0.5 N NaOH using phenophthalein as an indicator. This was made acidic with dilute HCl until the solution became

colourless and volume was made upto 50 ml. From this, duplicate aliquots of 20 ml were taken in centrifuge tubes, to which was added 4 ml of ferric chloride solution. The mixture was heated for 15 min in a boiling water bath, cooled and centrifuged. The supernatant was decanted and to the precipitate was added 5 ml of dilute HCI. It was again centrifuged and supernatant discarded. Two ml of double distilled water was added to the precipitate, heated for a few minutes in a boiling water bath followed by addition of 2 ml of 0.5 N NaOH and heated again for 15 min. The solution was filtered through Whatman no. 44 filter paper into a Kjeldahl's flask. Ferric hydroxide precipitate was washed well with hot water and washings added to the flask. One ml of concentrated sulfuric acid and 1 ml of perchloric acid were added. It was heated until all the organic matter was destroyed. The flask was cooled and the contents were poured into a 25 ml volumetic flask. The flask was washed several times and the washings added to the volumetic flask. Volume was then made up to the mark.

Estimation : Subsequently, phosphorus was determined by the method of Fiske and Subbarow (Oser, 1980), using 1,2,4 aminonapthol sulfonic acid (ANSA) as the colour reagent, as described later under the estimation of phosphorus in the meals.

When the above method was applied to estimate phytin-phosphorus in the diet samples, the estimated values were very low as compared to the food table calculated values. In order to test whether these low values were due to the method or actually low content of phytin-phosphorus in the diets, pure Na-phytate

was added in measured quantities to the diet samples and estimations repeated. The results revealed that there was 95% recovery of added Na-phytate using the present method. Even when wheat bran was analysed it gave results comparable to the calculated values. Similarly whole wheat, wheat flour (whole) and wheat flour (after seiving through 0.1 mm seive) gave expected values for phytin-phosphorus. Hence it was concluded that the above method could be used to estimate phytin-phosphorus content of diet samples with appreciable degree of accuracy.

Oxalate

Principle : Oxalate was extracted from the food sample with 2 N HCl and precipitated as calcium oxalate which was then titrated against standrd postassium permagnate solution (Baker, 1952). Oxalate is usually present in foods in a bound form, in combination with calcium, potassium or other salts. Its estimation from foods or cereal meals requires extraction with dilute acid and subsequent estimation. Only titrimetric methods are available for estimation of oxalic acid in foods. Therefore the method, as described by Baker (1952) has been used in the present study for estimating oxalate in cereal meals.

Extraction : Five gram sample of the diet powder was taken, to which 100 ml of 2 N HCl was added in a conical flask. This was shaken in a metabolic shaker for 2 hrs. The mixture was then centrifuged and filtered. The supernatant was transferred to the same flask and weighed. It was boiled for 15 min in a boiling water bath, cooled, reweighed and the loss in weight was made up

with double distilled water. The volume was made upto 100 ml with 2 N HCl, shaken well and filtered. To 25 ml of the filterate, 5 ml of phosphoric acid tungstate reagent was added, stirred well and kept overnight. The next day it was centrifuged and filtered. To 20 ml of the filterate, 2-3 drops of methyl red were added, neutralised with ammonia and 5 ml of calcium chloride buffer was then added and stirred well. The mixture was allowed to stand overnight, at the end of which it was filtered through Whatman no. 44 filter paper and washed free of chloride using double distilled water (as indicated by silver nitrate test).

Titration : The precipitate was transferred to the beaker along with the filter paper and some double distilled water was added, followed by 5 ml of 2 N H_2SO_4. The mixture was heated to 80°C over a burner and titrated against standard 0.01 N $KMnO_4$ solution until a faint pink colour persisted for 30 sec.

The oxalic acid content of the meals was calculated by using an equivalent of 0.45 mg of oxalate for every ml of 0.01 N $KMnO_4$, as indicated below :

$$\text{Oxalate in the meal (mg)} = \text{Titre value} \times 0.45 \times \frac{25}{20} \times \frac{100}{25} \times \frac{\text{Dry weight of the diet}}{5}$$

Calcium

Principle : Dry diet powders were ashed in a muffle furnace at 600 °C for 3-5 hr till constant weight and calcium was estimted in the ash solution by precipitation as calcium oxalate and subsequent titration against $KMnO_4$ as described by Clark and Collip (Oser, 1980).

Calcium can be estimated titrimetrically using $KMnO_4$ solution as well as colorimetrically using cresolphthalein dye in the presence of 8 quinolinol. However, for the purpose of estimating calcium in foods, titrimetric method is more suitable as it is quick, easy to carry out, less time consuming as well as feasible to carry out on large number of diet samples.

Precipitation : To two ml of the digested sample, 2 ml of double distilled water was added followed by 1 ml of 4% ammonium oxalate solution. The mixture was shaken and left overnight. The next day it was centrifuged for 5 min at 2000 rpm, the supernatant was drained off and the mouth of the centrifuge tube was wiped off with filter paper. Three ml of dilute ammonia was added to the precipitate and it was recentrifuged. This was repeated twice.

Titration : Two ml of 1 N H_2SO_4 was added to the ammonia washed precipitate. The tube was placed in a boiling water bath for 1 min and titrated against 0.01 N KMnO4 till a definite pink colour persisted for > 1 min.

The reading of a blank consisting of 2 ml of 1 N H_2SO_4 was subtracted from that of the sample. Calcium content of the diet was calculated using the following equivalent

1 ml of 0.01 N $KMnO_4$ = 0.2004 mg calcium

Phosphorus

The ash solution, prepared after dry ashing of the diet powders was used for estimation of phosphrus using the colorimetric method of Fiske and Subbarow (Oser, 1980).

Principle : Phosphorus containing sample solution is treated with molybdic acid to produce phosphomolybdic acid. This is reduced by the addition of 1,2,4 aminonapthol sulfonic acid (ANSA) reagent giving a blue coloured complex, the intensity of which can be read colorimetrically at 680 nm.

There are other micromethods available too, for estimating phosphate content in small quantities of the samples but these are more appropriate for biological samples such as serum, blood or urine analysis (Raghuramulu et al, 1983). For our purpose, where relatively larger quantities of diet powders were available, the colorimetric method as described by Fiske and Subbarow (Oser, 1980) was adequate to give a quantitative estimate of phosphorus in the meals.

Estimation : Suitable aliquots from the ash solution were taken in test tubes, to which 1 ml of molybdate II reagent was added. This was followed by addition of 0.4 ml of ANSA reagent. The volume was made upto 10 ml and the absorbance at 680 nm was

read between 9th and 10th minute after adjusting the instrument to zero with the reagent blank consisting of 5 ml of 10% trichloroacetic acid, 1 ml of molybdate II and 0.4 ml of ANSA and 3.6 ml of double distilled water.

Standard curve was constructed using standard phosphorus solution upto a concentration of 40 ug. Standards were treated in the same manner as the samples except that molybdate I reagent was added in the standards instead of molybdate II reagent for the samples.

Phosphorus content of the meal was calculated as follows :

$$\text{Phosphorus in the meal (mg)} = \frac{\text{Reading of Sample}}{\text{Reading of Standard}} \times \text{Conc. of Standard } (\mu g) \times \text{Dilution factor} \times \frac{1}{1000}$$

Proximate analysis

The proximate analysis of the diet was carried out on dry diet powder prepared by air oven drying as described earlier. Estimations for crude fat, protein, crude fibre and ash content of the diet were carried out by the methods of AOAC (1984). The content of available carbohydrate was then calculated by difference and the energy value of the diet was arrived at by taking into consideration the contributions of protein, fat and carbohydrates.

Fat : Crude fat was estimated as ether extract of the dry food material. For this 10 g of the dry diet powder was accurately weighed into a filter paper boat, which was then placed in a Soxhlet apparatus and extracted with anhydrous ether for about 16 hr. The ether extract was filtered into a weighed conical flask. The washings of the ether extract flask were added to the conical flask. The ether was removed by evaporation and the flask with the residue was dried in an air oven at 80-100 °C, cooled in a dessicator and weighed. Fat content of the diet was calculated as follows :

$$\text{Fat content of the meal (mg)} = \frac{\text{Weight of ether extract}}{\text{Weight of the sample}} \times \text{Dry weight of the diet}$$

Protein : Nitrogen content of the samples was estimated by the Kjeldahl method and by multiplying the total nitrogen content by 6.25, the protein content of the meals was obtained.

(a) **Principle :** For estimation of nitrogen, first all the nitrogenous material in the sample is converted to ammonium sulfate which is subsequently decomposed by addition of excess of alkali and liberated ammonia is absorbed into boric acid solution containing bromocresol green indicator by steam distillation. Ammonia forms ammonium borate which is titrated against standard sulfuric acid solution to give the total nitorgen content.

(b) Digestion : Two grams of the dry diet powder was accurately weighed and introduced into a long necked micro-kjeldahl flask and the sides were washed with double distilled water. A pinch of catalyst and 20 ml of concentrated H_2SO_4 was added and it was digested in the digestion chamber until charring began and white fumes appeared in the flask. Then the digestion was stopped for a while to cool the flask and a few drops of H_2O_2 were added and the digestion was continued till a colourless solution was obtained. The flask was allowed to cool.

(c) Distillation : Ten ml of boric acid solution was placed in a 100 ml conical flask and it was placed in such a way that the tip of the condenser outlet of the steam distilltion apparatus dipped below the surface of boric acid solution. Two to three drops of bromocresol green indicator were added. The digested food sample was transferred with repeated rinsings into the chamber of the steam distillation unit. To this, 8 ml of 40% NaOH was added and the steam generation was started and continued till about 30 ml distillate was collected into the receiving flask. The receiving flask was lowered and the steam generation was stoppped. The conderser outlet tube was washed with little distilled water into the receiving flask. The solution in the receiving flask was coloured blue at this time.

(d) Titration : The contents of the flask were titrated against 0.01 N H_2SO_4 solution till the original green colour was obtained.

For calculating the nitrogen content, the following factor was used :

1 ml of 0.01 N H_2SO_4 = 0.00014 g Nitrogen

Fibre : Crude fibre was estimated by acid and alkali treatment of the food sample respecitvely. For this, five g of moisture and fat free diet sample was taken in a 500 ml beaker and 200 ml of boiling 0.255 N H2SO4 was added. The mixture was boiled for 3 min keeping the volume constant by the addition of double distilled water at frequent intervals. The mixture was then filtered using a buchner funnel. The vacuum of the funnel was adjusted to 25 mm Hg (735 mm pressure). At the end of the filteration, 40-45 ml near boiling water was passed through the funnel to wash the residue. While adding wash water, the funnel was lifted from the apparatus to remove the vacuum.

The residue was washed from the funnel into the beaker with 200 ml of near boiling 0.313 N NaOH solution. This was boiled for 30 min, keeping the volume constant as described before. After this, it was filtered through the buchner funnel and the residue was washed with hot water as mentioned above.

The washed residue was transferred to a pre-wieghed crucible and dried at 80-100 °C in an air oven, cooled in a dessicator and weighed. The crucible was then ashed at 600 °C for 2 hr, cooled and weighed again. Crude fibre was calculated as shown below :

$$\text{Crude fibre (g) in the meal} = \frac{\text{Weight of residue before ashing} - \text{Weight of ash}}{5} \times \text{Dry weight of the diet}$$

Ash : Five gram of the dry diet powder was weighed into a pre-weighed, ignited and cooled crucible. This was placed in the muffle furnace at 550 to 600 °C (dull red) until light grey ash results. Crucible was cooled in a dessicator and weighed. To ensure complete ashing, the crucible was again heated for 1/2 hr in the furnace, cooled and re-weighed. This was repeated till two consecutive weights were the same and the ash was almost white or greysih white in colour. Ash content of the meal was calculated as follows :

$$\text{Ash content of the meal (mg)} = \frac{\text{Weight of ash}}{\text{Weight of the sample}} \times \text{Dry weight of the diet}$$

Carbohydrates : The content of available carbohydrates was determined by difference i.e by subtracting from the total dry weight of the diet, the sum of values (for the whole diet) for fat, protein, fibre and ash.

Statistical Analysis

Means and standard errors of four replicates for all parameters were calculated. Simple correlation was calculated between the in vitro estimated and the reported in vivo values for iron availability in the first phase of the study. The data

of the interaction effect of various enhancers and inhibitors in the pure system as well as from the standard meal was subjected to multiple regression analysis (stepwise technique), using the SPSS package on an IBM compatible PC assembly. The predictive powers of the two equations evolved, were compared using simple correlations between the computed and the analysed values for in vitro iron availability from typical Indian meals.