SUSTAIN

SHARING SCIENCE & TECHNOLOGY TO AID IN THE IMPROVEMENT OF NUTRITION

STORAGE, SENSORY, AND BIOAVAILABILITY EVALUATION OF IRON FORTIFIED CORN MASA FLOUR

FINAL REPORT
August 2000

This publication was made possible through the generosity of the the U.S. Agency for International Development and the Bill & Melinda Gates Foundation

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MISSION

The mission of SUSTAIN is to share science and technology to improve nutrition in developing countries. We do this by engaging industry, the scientific research community and governments in collaborative efforts to enhance the nutritive quality of food staples and by encouraging technologic innovation.

ORIGINS

SUSTAIN originated as a volunteer-based initiative to share food technology expertise with developing countries. SUSTAIN's early programs supported developing country food industries striving to improve product quality, food safety, packaging and marketing. SUSTAIN volunteers, drawn largely from U.S. food industries, provided requesting food companies with hands-on expertise to achieve these goals.

In the mid 1990's, SUSTAIN began to devote significant program attention to addressing the nutritional challenges of vulnerable populations. Our appreciation of the critical role micronutrients play in health and survival, particularly for infants, children, and women of childbearing age, led us to target applications of food science and technology to the pervasive problem of micronutrient deficiencies in developing countries.

BUILDING PARTNERSHIPS TO IMPROVE NUTRITION

In 1999, SUSTAIN launched operations as a 501(c)(3) non-profit organization whose goal remains technology sharing to improve global nutrition. SUSTAIN works as a catalyst organization, building partnerships across industry, the scientific and public health communities and government to improve the quality of food, and thus the quality of life for people in developing countries. SUSTAIN also sponsors research and encourages industry's development of innovative technologies in support of nutritional enhancements.

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ACKNOWLEDGEMENTS

SUSTAIN wishes to express its appreciation to all the individuals, institutions, and companies who helped make the storage study, sensory difference evaluation, and bioavailability study of iron fortified corn masa flour possible. The study was conducted with support from the U.S. Agency for International Development (USAID) and the generosity of the Bill and Melinda Gates Foundation, and in conjunction with the Laboratory for Human Nutrition, Institute of Food Science, Swiss Federal Institute of Technology (ETH), the Institute of Nutrition of Central America and Panama (INCAP), Monterrey Institute of Technology (ITESM), and Pacific Grain Products, Inc.

Our particular thanks and appreciation is extended to the research scientists involved in the study: Drs. Richard Hurrell and Lena Davidsson of ETH; Dr. Erick Boy, formerly of INCAP, Ms. Maria Leticia Bravo-Gutiérrez, M.S., of ITESM; and Mr. Neal Hammond, Pacific Grain Products, Inc. We would also like to recognize Mr. John Watson of Watson Foods and Mr. Wallace Yokohama, USDA, for their contributions to the sensory and storage studies, respectively. Mr. Ezequiel Montemayor, Dr. Guillermo Arteaga, Dr. Miguel Arce, and Dr. Fernando Ramirez, all from MASECA also provided invaluable input and support to these studies, including donating the corn masa flour samples. Gratitude is also owed to Dr. Sam Kahn of USAID and SUSTAIN's corn masa flour advisory panel members from industry, academia, and government for volunteering key assistance and guidance to this study (see Appendix 1).

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This publication was made possible through funding provided by United States Agency for International Development (Cooperative Agreement No. DAN-A-00-12-00066-06) and the generosity of the Bill and Melinda Gates Foundation. The opinions expressed herein are those of the author(s) and do not necessarily reflect the views of the United States Agency for International Development.

EXECUTIVE SUMMARY

The recent development of industrially produced corn masa flour and its increasing popularity in Mexico and Central America has created the opportunity for using corn masa flour as an iron fortification vehicle for the first time. This has the potential to greatly impact the nutritional status of millions of people vulnerable to iron deficiency anemia (IDA), which may be prevented though iron fortification. IDA is a major public health concern in developing countries, particularly in many parts of Latin America where corn is a widely consumed staple food.

In 1998 SUSTAIN commissioned a three-part study to help identify an iron fortificant for use in corn masa flour that is absorbable by the body and which does not adversely affect the product. Elemental iron has been used for wheat flour fortification for nearly 50 years; however, some studies have shown that its bioavailability is lower than other iron forms. For this reason, SUSTAIN evaluated alternative iron compounds, namely ferrous sulfate and fumarate with and without an iron absorption enhancer, disodium-EDTA (Na_2EDTA). The research included a fat oxidation study, a sensory difference evaluation, and a bioavailability study. The fat oxidation study assessed the iron compounds' ability to provoke rancidity in corn masa flour, while the sensory difference evaluation was conducted to determine whether consumers could detect a difference in corn masa flour tortillas fortified with the various iron fortificants. It was not asked whether this difference was acceptable or not. The sensory study was conducted in two phases: in phase I consumers sampled tortillas that were freshly fortified while in phase II consumers evaluated flours that were fortified two months before. The stable-isotope study measured iron bioavailability in healthy Guatemalan females. The study was commissioned by SUSTAIN and conducted with support from the U.S. Agency for International Development (USAID). Research activities were carried out in conjunction with the Laboratory for Human Nutrition, Institute of Food Science, Swiss Federal Institute of Technology (ETH), the Institute of Nutrition of Central America and Panama (INCAP), Monterrey Institute of Technology (ITESM), and Pacific Grain Products, Inc.

The fat oxidation studies indicated that, in this case, iron fortification of corn masa flour does not create more pronounced rancidity problems in the product upon storage as compared with non fortified corn masa flour. The sensory difference evaluation confirmed that tortillas made from corn masa flour fortified with elemental iron were the most similar to the control (no iron added) in phase I and II evaluations. Differences were detected, however, in tortillas fortified with ferrous fumarate, ferrous sulfate (in both cases after two months storage), sodium-iron-EDTA [(NaFeEDTA), phases I and II], and ferrous fumarate plus Na_2EDTA (phase I). The human bioavailability studies showed that NaFeEDTA demonstrated the best iron absorption (geometric mean iron absorption 9% compared to 5.5% from ferrous sulfate). Fractional iron absorption from NaFeEDTA is especially significant given the high phytate content of the test meal (corn tortillas and black bean paste), which is a staple meal in many regions of Latin America. Ferrous fumarate also performed well, with a relative bioavailability value that ranged from 5.5 to 6.7%--similar to that of ferrous sulfate. When Na_2EDTA was added to ferrous fumarate, however, its influence on absorption was not statistically significant. Elemental iron was not evaluated in the bioavailability study.

In conclusion, ferrous fumarate had the best overall performance of all the iron compounds evaluated in corn masa flour based on its sensory, storage, and bioavailability results. From the bioavailability perspective only, NaFeEDTA was the superior candidate while from a sensory standpoint elemental iron fared the best. However, the bioavailability of elemental iron is not fully understood, nor is the effect of NaFeEDTA and ferrous fumarate on the sensory characteristics of corn masa flour. To further examine these issues as well as cost-effectiveness concerns, additional research is needed.

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I. INTRODUCTION

Iron (Fe) deficiency anemia is a major nutritional problem in the world, particularly in vulnerable population groups such as infants, children, and women of childbearing age (DeMaeyer & Adiels-Tegman 1985, Bothwell *et al.* 1979). Strategies to combat Fe deficiency include supplementation with medicinal Fe as well as fortification of foods with Fe. Food fortification programs are generally considered the most cost-effective approach (Hurrell 1997, 1998), costing about $1 per affected person per year (World Bank, 1994). Some food vehicles used in Fe fortification (staples such as cereal flour, salt and sugar) reach the general population whereas other foods are targeted at specific at-risk groups. Fe fortification of infant formula, for example, has been successful in reducing the prevalence of Fe deficiency in infants (Dallman 1990).

The overall success of Fe fortification programs depends on several factors. One of the most important factors is the relationship between Fe bioavailability and the organoleptic, or sensory, changes in the fortified food(s). For instance, water-soluble Fe compounds such as ferrous sulfate have high relative bioavailability yet they may have an undesirable organoleptic impact on the product, i.e. provoking rancidity or undesirable color and flavor changes during storage or food preparation. In order to minimize such organoleptic changes, many fortification programs use less soluble, and hence less bioavailable Fe compounds, such as elemental Fe and Fe phosphate compounds (Forbes *et al.* 1989).

Thus, there is a need for alternative Fe compounds that possess high relative bioavailability which do not provoke unacceptable organoleptic changes in cereal foods. Ferrous fumarate has been proposed as an alternative for Fe fortification of infant cereals since this compound was shown to be absorbed as well as ferrous sulfate in adults (Hurrell *et al.* 1989). (Ferrous sulfate is the iron compound generally used as the standard by which to measure the relative bioavailability of other iron forms). Ferrous fumarate is less soluble than ferrous sulfate in water but is readily soluble in dilute acid such as gastric juice. Further, ferrous fumarate does not provoke unacceptable organoleptic changes in the food during storage to the same extent as does ferrous sulfate and it has a high relative bioavailability. Therefore, ferrous fumarate could be a very promising Fe fortificant for food fortification of cereal products such as corn masa flour.

Bioavailability of dietary Fe (including added Fe) depends on the overall composition of the meal, i.e., the presence of enhancers and inhibitors of Fe absorption. Corn masa flour contains relatively high levels of phytic acid, a potent inhibitor of Fe absorption. Fe bioavailability from fortified corn masa could therefore be expected to be relatively low, unless an absorption enhancer is added. Ascorbic acid is one of the major enhancers of Fe absorption. Its positive effect on Fe absorption has been demonstrated in several studies of adults (Gillooly *et al.* 1984, Hallberg *et al.* 1989, Siegenberg *et al*. 1991) as well as in infants and school children (Stekel *et al*. 1986, Davidsson *et al*. 1994, 1998a, b).

Ascorbic acid is often used in commercial food products as an enhancer of Fe absorption as well as an antioxidant in processed foods. However, due to its susceptibility to oxidation by exposure to air, particularly when the food is also exposed to heat and/or moisture, the usefulness of ascorbic acid as an enhancer of Fe absorption can be questioned in some situations.

Consequently, a more stable enhancer of Fe absorption is needed, especially in developing countries where the storage and packaging of food can be a serious issue. (INACG 1993).

Disodium-EDTA (Na_2EDTA) is another iron absorption enhancer. This compound has been demonstrated to increase Fe absorption two- to three-fold relative to ferrous sulfate from meals high in phytic acid (INACG 1993) and appears to protect against fat oxidation during storage of cereal products (Hurrell 1997). It is stable during normal food processing and storage and is a permitted food additive in specified foods. The acceptable daily intake (ADI) for EDTA is 2.5-mg/kg body weight (JECFA 1974). The enhancing effect of Na_2EDTA on Fe absorption in humans has been demonstrated previously in studies with low bioavailability meals. Fe absorption from Egyptian bread fortified with ferrous sulfate was significantly increased after the addition of Na_2EDTA at a molar ratio of 1 relative to Fe (El Guindi *et al*. 1988). A recent study demonstrated that adding Na_2EDTA at molar ratios of 0.25-1.0 relative to Fe significantly increased Fe absorption from a rice-based test meal (MacPhail *et al.* 1994). In addition, a significant enhancing effect on Fe absorption was achieved by adding Na_2EDTA at molar ratios of 0.33, 0.69, and 1 relative to Fe to a cereal based meal fortified with ferrous sulfate in 6-7 year old children (Davidsson *et al.* 1998b). Until the present study, no information was available on the effect of Na_2EDTA on Fe bioavailability of other Fe compounds such as ferrous fumarate.

To further explore the potential for improving nutrition through fortification of cereal foods with iron, SUSTAIN commissioned a three-part study to help identify an iron fortificant for use in corn masa flour that is absorbable by the body and does not adversely affect the product. This study is particulary relevant given that corn masa flour is now produced on a commercial scale and corn is a widely consumed staple food in many parts of Latin America. Elemental iron has been used for wheat flour fortification for nearly 50 years; however, some studies have shown that its bioavailability is lower than other iron forms. For this reason, SUSTAIN evaluated alternative iron compounds, namely ferrous sulfate, ferrous fumarate (with and without added Na_2EDTA) and NaFeEDTA. The research included a fat oxidation study, a sensory difference evaluation, and a bioavailability study. The fat oxidation study assessed the iron compounds' ability to provoke rancidity in corn masa flour after storage. The sensory difference evaluation was conducted to determine whether consumers could detect a difference in corn masa flour tortillas fortified with the various iron fortificants. The stable-isotope study measured iron bioavailability in healthy Guatemalan females.

SUSTAIN conducted the research with support from the U.S. Agency for International Development (USAID) in conjunction with the Laboratory for Human Nutrition, Institute of Food Science, Swiss Federal Institute of Technology (ETH), the Institute of Nutrition of Central America and Panama (INCAP), Monterrey Institute of Technology (ITESM), and Pacific Grain Products, Inc. Detailed information on the three studies is captured in the following two sections, written by the principal investigator for each case. The last section offers a summary and conclusions from the studies and proposes next steps. In its entirety, the final report is intended to augment the current body of knowledge on the use of iron fortificants in corn masa flour with the goal of improving the iron status of vulnerable groups across the developing world.

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II. STORAGE AND SENSORY EVALUATION OF IRON FORTIFIED CORN MASA FLOURS

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Introduction

Iron compounds are difficult to add to cereal-based foods since they can provoke rancidity and adverse color and flavor changes (Hurrell 1997). To further explore the reactions between iron fortificants and cereals, SUSTAIN commissioned storage and sensory studies to evaluate iron compounds in fortified corn masa flour. These studies were funded by the U.S. Agency for International Development (USAID) and conducted by the Laboratory for Human Nutrition, Institute of Food Science, Swiss Federal Institute of Technology (ETH), Monterrey Institute of Technology (ITESM), and Pacific Grain Products, Inc. Two methods were used to investigate the ability of the iron fortification compounds to provoke fat oxidation in corn masa flour during storage. Fat oxidation was measured by pentane production over a four-month storage period at 37^{o}C at the Nestlé R&D Center, Switzerland (Hurrell *et al.* 1989) and by a rapid method of hexanal production after 16 hours at 65^{o}C (Pacific Grain Products Inc., Hammond). In the sensory study, tortillas were prepared from the iron-fortified corn masa flours and a consumer panel was asked to evaluate differences between tortillas prepared with and without various iron compounds (Bravo-Gutiérrez, ITESM). This consumer study was designed to determine whether differences occurred, but not whether the differences were acceptable to the consumer.

Fat Oxidation Study

A series of different iron compounds were supplied by Watson Foods Company, CT, dry-mixed into corn masa flour at 10mg Fe/100g, and analyzed at laboratories in Switzerland (for pentane) and California (for hexane).

Pentane measurements
Corn masa flour [8.8% moisture, 1.39% ash, 3.6% fat, 11.9% protein (N x 5.7)] was weighed as 25g aliquots in 1L metal cans. The cans were sealed and stored at 37^{o}C for 4 months. Each month, pentane and oxygen was measured in the headspace by gas chromatography and the sensory quality was evaluated by sniffing the odor.

The results are shown in Table 1. Sample R contained ferric nitrate and was selected to provoke fat oxidation; sample C was the control without any addition of iron. Sample R generated pentane after only 1 month, with the level increasing progressively over the four-month period. During this time the oxygen concentration was reduced drastically. After one month, the sample was strongly oxidized and had unacceptable sensory attributes. The control sample with no added iron was stable for 2 months but had oxidized at 3 months and 4 months, generating pentane and giving unacceptable odor scores. All the iron fortified samples behaved in a similar way to the control sample, oxidizing between 2 and 3 months of storage. There were no differences between ferrous sulfate, ferrous fumarate, (both with and without Na_2EDTA), and NaFeEDTA.

We can conclude that all corn masa flour used in this study had poor stability. It was stable for only 2 months at 37^oC, which can be extrapolated to 4 months at 30^oC or 8 months at 20^oC under these standardized conditions. The iron compounds tested did not affect keeping quality.

Hexanal measurements
The same iron-fortified corn masa flours that were evaluated in the pentane test were evaluated by the more rapid hexanal test after 16 hours heating at 65^oC of 50g aliquots in sealed 4 oz glass bottles, and also after storage in same glass bottles for 3 months at room temperature. The results after 16 hours heating are shown in Table 2.

The ferric nitrate-fortified corn masa produced 3.2 times more hexanal than the control sample with no added iron. The levels of hexanal produced from the iron-fortified corn masa flours were somewhat inconsistent and ranged from 40-140% of the control value. There was no clear evidence, however, that any of the test iron compounds, or Na_2EDTA, influenced hexanal formation. Similarly, after the samples had been stored for 3 months at room temperature, no hexanal was detected in any of the stored corn masa flours (except that containing ferric nitrate) or in the control flour. These results are not consistent with the pentane data after storage, for unknown reasons. Hexanal was detected in the stored corn masa flour with added ferric nitrate at a level similar to that generated when the same sample was heated for 16 hours at 65^oC. This indicates that in a highly pro-oxidant form, iron readily catalyzes fat oxidation of corn masa.

From these studies, there is no evidence that any of the added iron fortification compounds or Na_2EDTA will influence fat oxidation or reduce the shelf life of stored corn masa flour.

Sensory Difference Evaluation

Test results compiled by Maria Leticia Bravo-Gutiérrez of ITESM on 'Sensory Differences in Iron-Fortified Tortillas' were submitted to SUSTAIN in April 1999. The following is a brief description and evaluation of the reported findings.

Five different batches of iron-fortified corn masa flour plus the control were tested. Ferrous sulfate, ferrous fumarate and NaFeEDTA were added to corn masa flour at 4mg Fe/100g.[1] Ferrous fumarate was added at 4mg Fe/100g together with Na_2EDTA at a 1:1 molar ratio, while elemental iron was added at 3.5mg Fe/100g. Consumers were asked to evaluate differences in tortillas (in sample sets of three), which were made from fortified corn masa flour. There were two phases of evaluations, 2 months apart, during which participants were asked to evaluate tortillas made from freshly fortified flour (phase I), and then from flour stored for two months

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[1] 10mg Fe/100g was used in the fat oxidation studies and 4mg Fe/100g in the absorption studies. The higher level in the fat oxidation studies was an attempt to accelerate any possible sensory changes.

(phase II). The flour was stored for two months in 1-kg paper sacks at 25°C and 65% relative humidity.

On the day prior to the taste panel evaluation, tortillas (15 x 0.3cm) were made using a manual tortilla machine, cooked and then refrigerated. On the day of the study, they were reheated and fed to the subjects. Eleven to 25 panelists of Central American and Panamanian origin participated in the five sessions to evaluate the freshly fortified flours, and 16 to 19 panelists evaluated the stored flours. A triangle test was used (Lyon *et al.* 1992; Pedrero & Pangborn 1989). This triangle study required consumers to judge which of the three samples were different and to comment on the difference. The study did not solicit which samples were more acceptable.

Results and Discussion

From both the fat oxidation and sensory difference evaluations, it can be concluded that short-term storage (2 months at 25°C) of iron-fortified corn masa flour does not cause unacceptable fat oxidation reactions. In both phases, no differences were detected when the following sets of tortillas were compared: control (no fortification) versus reduced iron; ferrous fumarate versus ferrous fumarate plus Na_2EDTA; ferrous fumarate versus reduced iron; ferrous fumarate plus Na_2EDTA versus reduced iron. However, differences were detected in both phases for the following combinations: control versus NaFeEDTA; ferrous fumarate versus NaFeEDTA; ferrous fumarate plus Na_2EDTA versus ferrous sulfate; and ferrous sulfate versus reduced iron. Statistically significant differences in sensory quality between the control and the iron-fortified tortillas are shown in Table 3. Elemental iron scored the best among consumers as no significant difference was detected in comparison to the control in either phase.

In addition to the difference evaluation, participants were also encouraged to provide additional qualitative comments related to color, flavor, and texture. While the qualitative comments were not formally solicited as part of the difference evaluation protocol, they did provide some interesting information. As might be expected, no negative comments were provided on elemental iron. However, there were some negative comments with regard to color, taste and texture properties for ferrous sulfate- and NaFeEDTA-fortified tortillas. Participants also commented that all tortillas made from flour stored for two months, even those made with the control flour, had some negative texture characteristics. To further explore these comments, we recommend that additional sensory evaluations be conducted on NaFeEDTA and ferrous fumarate in tortillas made from fortified corn masa flour.

Table 1.
Sensory Evaluation Pentane Study

		Pentane (ppm)					Residual Oxygen (%)					Odor Marks				
Sample	**Description**	**1**	**2**	**3**	**4**	**6**	**1**	**2**	**3**	**4**	**6**	**1**	**2**	**3**	**4**	**6**
		months					**months**					**months**				
1	ferrous sulphate	0.51	0.90	32.6	58.6		20.9	20.6	13.2	10.4		7.3	6.3	3.6	4.0	
2	ferrous fumarate	0.41	0.74	29.5	59.7		20.7	20.5	13.5	9.8		7.3	6.3	3.3	3.6	
3	NaFeEDTA	0.40	0.68	15.2	47.3		20.9	20.6	15.7	11.4		7.3	6.3	4.3	3.0	
4	ferrous sulphate + Na_2EDTA (1:1)	0.53	0.84	26.8	58.6		20.7	20.4	13.5	9.8		7.3	6.3	4.3	3.0	
5	ferrous sulphate + Na_2EDTA (1:0.5)	0.45	0.93	7.43	44.5		20.9	20.7	17.9	11.7		7.3	6.3	4.6	3.0	
6	ferrous fumarate + Na_2EDTA (1:1)	0.37	0.72	46.5	43.2		20.9	20.5	11.1	14.5		7.3	6.3	3.3	3.0	
7	ferrous fumarate + Na_2EDTA (1:0.5)	0.45	0.82	36.8	53.7		20.9	20.5	12.2	10.4		7.3	6.0	3.3	3.0	
R	ferric nitrate	2.71	41.3	76.8	91.0		19.8	12.2	9.2	8.0		4.3	4.0	3.6	3.3	
C	control – no added iron	0.44	0.78	27.1	51.5		20.6	20.6	13.7	10.8		7.3	6.3	4.3	3.0	

Sample size: 25g in 1000cc cans; storage at 37º C
Odor marks: 9= fresh, perfect sample; 5=limit of acceptability; 1=extremely oxidized

Table 2.
Sensory Evaluation Hexanal Study

Sample	Description	Relative Hexanal Production*
1	ferrous sulfate	49
2	ferrous fumarate	ND**
3	NaFeEDTA	64
4	ferrous sulfate + Na_2EDTA (1:1)	115
5	ferrous sulfate + Na_2EDTA (1:0.5)	82
6	ferrous fumarate + Na_2EDTA (1:1)	136
7	ferrous fumarate + Na_2EDTA (1:0.5)	53
R	ferric nitrate	322
C	control – no added iron	100

*Hexanal content in test samples is reported relative to hexanal content in the control sample, as quantified by the size of the chromatographic peak
**ND=not determined

Table 3.
Statistically Significant Differences in Taste Between Iron Fortified Tortillas and the Control*

Iron Compounds Compared	Tortillas made from freshly-fortified CMF	Tortillas made from CMF stored 2 months @ 25^o
Control vs. ferrous sulfate	NS**	p=0.001
Control vs. ferrous fumarate	NS	p=0.05
Control vs. NaFeEDTA	p=0.01	p=0.04
Control vs. ferrous fumarate plus Na_2EDTA	p=0.005	NS
Control vs. elemental iron	NS	NS

*Measured by a triangle test
**NS=not significant

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III. IRON BIOAVAILABILITY FROM IRON FORTIFIED CORN MASA TORTILLAS

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Introduction

Corn masa flour has a high phytate level that compromises the body's ability to absorb dietary iron (Fe), including fortification Fe. This SUSTAIN-commissioned study was designed to evaluate Fe bioavailability from test meals based on corn masa flour tortillas and black bean paste fortified with ferrous sulfate, ferrous fumarate (with and without added Na_2EDTA) and NaFeEDTA. Corn masa flour was fortified with ferrous fumarate with and without Na_2EDTA, ferrous sulfate, or NaFeEDTA (including fortification iron). Corn masa flour, often served as tortillas, is a major staple food in many Central American countries, particularly among rural populations (Bressani *et al.* 1997). Thus, this type of flour could be a very useful vehicle for general Fe fortification programs in countries such as Guatemala and Mexico. The research was supported by USAID and conducted by the Laboratory for Human Nutrition, Swiss Federal Institute of Technology (ETH), and the Institute of Nutrition of Central America and Panama (INCAP).

Corn masa flour contains relatively high levels of native Fe, approx. 3-4 mg/100 g, and the addition of Na_2EDTA would be expected to increase Fe absorption of both native Fe and Fe added as a food fortificant. The level of Fe fortification in this study, 4 mg Fe/100 g, would increase the total Fe content to approx. 7-8 mg Fe/100 g fortified corn masa flour. Based on a daily intake of 250-500 g corn masa flour, and 5 % Fe absorption from Fe-fortified corn masa flour containing added Na_2EDTA, approx. 0.9-2.0 mg Fe would be absorbed per day from fortified corn masa flour. Consumption of Fe fortified corn masa flour with added Na_2EDTA would therefore be expected to have a significant impact on Fe nutrition in the population.

The addition of Na_2EDTA at a 1:1 molar ratio relative to total Fe (Study 1) and relative to fortification Fe (Study 2) was evaluated in corn masa tortillas fortified with ferrous fumarate. In Study 3, Fe bioavailability from ferrous sulfate was compared with NaFeEDTA. The impact of added Na_2EDTA on Fe bioavailability from the test meals (Studies 1-2) or Fe bioavailability from ferrous sulfate versus NaFeEDTA (Study 3) was evaluated by paired comparisons with each study subject acting as her own control. The Fe isotopic composition of whole blood samples was measured according to the recently developed technique by Walczyk (1996), using a Finnigan MAT 262 thermal ionization mass spectrometer (Finnigan MAT, Bremen, Germany).

Study subjects
Dr. Boy (INCAP) coordinated the study logistics and identified study participants. Apparently healthy adolescent Central American girls (12-13 years, max. body weight approx. 40 kg; 11

girls per study, total 33 girls) were recruited at public high schools in Guatemala City. Girls consuming Fe supplements or medication were excluded.

Subjects and parents were fully informed of the goals and procedures of the study and written consent was obtained from at least one parent. The Ethical Committees at INCAP, Guatemala City and the ETH, Zürich reviewed and approved the protocol. A physician (Dr. Boy) was available during blood sampling and administrations of labeled test meals. Remuneration for participation in the study was school materials (school bag and/or books, pens; total value US$15.00).

Materials and Methods

Test meals
Test meals were representative of a typical Central American meal based on Fe-fortified corn masa tortillas (made from 50 g corn masa flour), served with refried black bean paste (50 g) and water (250 g deionized water). Test meals were prepared from local raw materials, purchased in bulk. Each test meal contained 2-mg added ^{57}Fe (as labeled ferrous fumarate, ferrous sulfate or NaFeEDTA). Refried black bean paste was spread on the tortillas (2 tortillas per serving). Fe stable isotopes (and Na_2EDTA) were added to the bean paste, the tortillas were folded and heated in a microwave oven before being served. All test meals were fed twice a day for two consecutive days as breakfast and lunch (four test meals in total).

Girls were randomized to studies 1, 2, or 3. Further randomization was done within each study: five to six girls started with test meal A and five-six girls with test meal B. The alternate test meal was administered 14 days later. No food or drink was allowed for 3 hours after intake of the labeled test meals.

<u>Study 1.</u>

A. Corn masa tortillas and black bean paste fortified with ferrous fumarate (^{57}Fe-ferrous fumarate)
B. Corn masa tortillas and black bean paste fortified with ferrous fumarate (^{57}Fe-ferrous fumarate)
   Na_2EDTA: Fe molar ratio 1:1 [relative to total Fe in fortified corn masa flour (approx. 4 mg Fe)]

<u>Study 2.</u>

A. Corn masa tortillas and black bean paste fortified with ferrous fumarate (^{57}Fe-ferrous fumarate)
B. Corn masa tortillas and black bean paste fortified with ferrous fumarate (^{57}Fe-ferrous fumarate)
   Na_2EDTA: Fe molar ratio 1:1 [relative to fortification Fe in fortified corn masa flour (2 mg Fe)]

Study 3.

A. Corn masa tortillas and black bean paste fortified with ferrous sulfate (^{57}Fe-ferrous sulfate)
B. Corn masa tortillas and black bean paste fortified with NaFeEDTA (^{57}Fe-ferric chloride mixed with Na_2EDTA, molar ratio Fe: Na_2EDTA 1:1, added as a solution)
Served with: Black beans

Study design
Day 1: Blood samples were drawn after an overnight fast. Intake of labeled test meals (A or B) as breakfast (after an overnight fast) and again as lunch under standardized conditions (test meals were administered in the order AAAA or BBBB).

Day 2: Intake of labeled test meals (A or B) as breakfast (after an overnight fast) and again as lunch under standardized conditions.

Day 16: Blood samples were drawn after an overnight fast. Intake of labeled test meals (A or B) as breakfast (after an overnight fast) and again as lunch under standardized conditions (test meals were administered in the order BBBB or AAAA).

Day 17: Intake of labeled test meals (B or A) as breakfast (after an overnight fast) and again as lunch under standardized conditions.

Day 31: Blood samples were drawn after an overnight fast.

Stable isotope labels
The preparation of ^{57}Fe-ferrous fumarate (as a dry powder) was done in collaboration with one of the major commercial suppliers of Fe fortification compounds; Dr. P. Lohmann, Emmerthal, Germany, according to the industrial production of ferrous fumarate using a small-scale laboratory procedure. Its physico-chemical properties were tested and found to be similar to the commercial compound. This material has been used in previous human studies (Davidsson *et al.* 2000).

Solutions of $^{57}FeSO_4$ and $^{57}FeCl_3$ were prepared from isotopically enriched elemental Fe by dissolution in 0.1 M H_2SO_4 or HCl. The solution of $^{57}FeSO_4$ was stored in Teflon containers flushed with argon to keep the Fe in the +II oxidation state. The isotopic composition of the stable isotope labels was determined by negative thermal ionization mass spectrometry using a magnetic sector field instrument (MAT 262). Fe concentration of the labels was determined by isotope dilution mass spectrometry against a standard prepared gravimetrically from an Fe isotope reference material.

Na_2EDTA doses
Water solutions of Na_2EDTA [$Na_2EDTAx2H_2O$ (Merck)] were prepared immediately before administration and added to the test meals at the time of serving. Labeled NaFeEDTA was prepared immediately before administration by mixing solutions of ^{57}Fe-ferric chloride and Na_2EDTA (molar ratio Fe: Na_2EDTA 1:1).

Blood samples

Venous blood samples (5 ml) were drawn in EDTA-treated tubes before the first administration of the labeled test meal and again on Days 16 and 31. Samples were analyzed for Fe status indices (Hb, plasma-ferritin) and for the incorporation of ^{57}Fe into red blood cells. Blood samples were aliquoted for the analysis of Hb and plasma was separated, aliquoted and frozen for later analysis of ferritin. Hb was measured by the cyanmethemoglobin method (Sigma kit, Sigma, St. Louis, MO) and plasma-ferritin by ELISA kits (Ramco Laboratories, Houston, Texas). Commercial quality control materials (DiaMed, Cressier sur Morat, Switzerland and Ramco) were analyzed together with all series of samples analyzed for Hb and plasma ferritin.

Analysis of isotopic composition of blood samples

Each isotopically enriched blood sample was analyzed in duplicate for its Fe isotopic composition under chemical blank monitoring. Whole blood samples were mineralized using an HNO_3/H_2O_2 mixture and microwave digestion. This was followed by separation of the sample Fe from the matrix by anion-exchange chromatography, which came after a solvent/solvent extraction step into diethylether (Beer & Heumann 1993, Kastenmayer *et al.* 1994, Walczyk *et al.* 1997). All isotopic analysis was performed by negative thermal ionization mass spectrometry (NTI-MS) using a magnetic sector field mass spectrometer (MAT 262) equipped with a multi-collector system for simultaneous ion beam detection (Walczyk 1996). Fe separated from the sample was loaded on BaF_2 coated rhenium-filaments of a double-filament ion source together with AgF to promote the formation of negatively charged FeF_4^- ions. Because of the high enrichment of the isotopically enriched labels and the low amounts of isotopic label incorporated into the red blood cells, data were normalized for the natural $^{54}Fe/^{56}Fe$ isotope ratio (Taylor *et al*. 1992).

Calculation of Fe absorption

The amounts of ^{57}Fe isotopic label present in the blood 14 days after test meal administrations were calculated based on the principles of isotope dilution and considering that the Fe isotopic labels were not monoisotopic (Walczyk *et al.* 1997). This took into account the shift of the Fe isotope ratios in the blood samples and the amount of Fe circulating in the body. Circulating Fe was calculated based on blood volume and Hb concentration (Kastenmayer *et al*. 1994). Blood volume calculations were based on height and weight according to Brown *et al*. (1962). For calculations of fractional absorption, 80% incorporation of the absorbed Fe into red blood cells was assumed. Corrections for enriched baseline values were made when calculating Fe absorption from the second test meal.

Food analysis

Food samples were mineralized by microwave digestion in a HNO_3/H_2O_2 mixture and analyzed for Fe and calcium by electrothermal/flame atomic absorption spectroscopy (SpectrAA 400, Varian, Mulgrave, Australia) using standard addition technique to minimize matrix effects. Phytic acid content was determined by a High Performance Liquid Chromatography (HPLC) technique (Sandberg & Ahderinne 1986, Sandberg *et al*. 1989).

Statistics
Paired t-test was used to evaluate data within each study. Values were logarithmically transformed before statistical analysis. Results are presented as geometric means (+1SD and –1SD).

Results

Test meals contained 2.2 mg native Fe, 58.3 mg calcium and 427 mg phytic acid (IP 5-6). None of the girls had Hb concentration < 120 g/L. Geometric mean plasma-ferritin was 22 μg/L at baseline and 19 μg/L at Day 14. Four girls had no Fe stores, as indicated by plasma ferritin < 12 μg/L. Individual data on Hb, ferritin and Fe absorption are given in Tables 4-6.

Results from this study indicate that the influence of Na_2EDTA on Fe absorption from ferrous fumarate was not statistically significant (p=0.5). In Study 1, geometric mean Fe absorption (+1SD, -1SD) was 5.5 % (14.8, 2.0; no added Na_2EDTA) and 6.7 % (14.2, 3.1; added Na_2EDTA). The geometric mean Fe absorption in Study 2 was 6.2 % (10.8, 3.6) and 5.8 % (11.5, 2.9) from test meals without added Na_2EDTA and after addition of Na_2EDTA, respectively.

Geometric mean Fe absorption from test meals fortified with ferrous sulfate (test meal A, Study 3) was 5.5 % (16.5, 1.8). Fe absorption from ferrous sulfate upon addition of NaFeEDTA increased to 9% (25.5, 3.2) and was statistically significant (p=0.009).

Discussion

Previous studies have clearly demonstrated that Fe bioavailability from ferrous sulfate can be significantly increased by adding Na_2EDTA to inhibitory, cereal-based meals (El Guindi *et al*. 1988, MacPhail *et al.* 1994, Davidsson *et al.* 1998b). This enhancing effect on Fe absorption could not be confirmed in the present study with ferrous fumarate. Fe bioavailability from ferrous fumarate was not significantly influenced by the addition of Na_2EDTA at a molar ratio 1:1 relative to fortification Fe or relative to the total Fe content in the corn tortillas. The discrepancy in the effect of Na_2EDTA on a water soluble Fe compound (ferrous sulfate) and a compound which is soluble in dilute acid (ferrous fumarate) indicates the importance of careful evaluations of Fe absorption enhancers on compounds with different physico-chemical properties. Na_2EDTA's lack of effect on ferrous fumarate absorption can be assumed to be related to the solubility properties of this Fe compound in the gastric juice and the complex formation between the EDTA moiety, Fe, and other minerals and trace elements present in the meal.

Although no direct comparisons between ferrous fumarate and ferrous sulfate were made in this study, the data indicate that fractional Fe absorption from the test meal fortified with ferrous fumarate was similar to that from ferrous sulfate—the Fe compound which is generally set at 100% relative bioavailability. This is in agreement with the earlier study in adults by Hurrell *et al.* (1989). Relatively high fractional Fe absorption was found in the current study, (geometric mean values 5.5-6.7 %) most likely due to the fact the test subjects were young girls with limited stored Fe and high requirements for absorbed Fe. For comparison, Fe absorption from test meals based on corn flour has been reported to be lower in adults (Cook *et al.* 1997, Bressani *et al.* 1997).

The traditional Central American meal based on corn and black beans used in this study contained a relatively high amount of phytic acid (427 mg) and no enhancers of Fe absorption such as animal tissue or ascorbic acid. Thus, Fe absorption from this meal can be expected to be low unless the Fe is protected against the inhibitors of Fe absorption. The results from Study 3 clearly show that Fe absorption from ferrous sulfate was inhibited by the composition of the test meal. The increase in Fe absorption when NaFeEDTA was used as the Fe fortificant was statistically significant.

The alternative Fe compound NaFeEDTA has been suggested for fortification of cereal products (Hurrell 1998) due to its high Fe absorption level in meals with high phytic acid content (INACG 1993). The results from the present study confirm the usefulness of NaFeEDTA as the Fe fortificant for staple foods with high content of phytic acid such as corn masa flour. NaFeEDTA is not currently used in large-scale food fortification programs. However, the recent evaluation by the Joint FAO/WHO Expert Committee on Food Additives concluded that NaFeEDTA can be considered to be safe when used in supervised food fortification programs, providing approximately 0.2 mg Fe/kg body weight per day (JECFA 1999). The use of this Fe compound will presumably increase in the near future.

In conclusion, Fe absorption was significantly enhanced from this inhibitory meal when ferrous sulfate was replaced by NaFeEDTA. No enhancing effect on Fe bioavailability was found after addition of Na_2EDTA to a typical Guatemalan meal based on corn tortillas and black bean paste fortified with ferrous fumarate. The nutritional impact of a food fortification program using ferrous fumarate as the Fe fortificant would not be increased by the addition of Na_2EDTA. Thus, the results from this bioavailability study support the use of NaFeEDTA for food fortification of inhibitory staple foods such as corn masa flour.

Table 4.
Bioavailability Study 1*

ID	Hb (g/L) (A)	Ferritin (μg/L) (A)	Fe absorption (%) (A)	Fe absorption (%) (B)	Hb (g/L) (B)	Ferritin (μg/L) (B)
8	132.7	28	2.4	8.2	129.0	20
10	146.9	28	10.9	2.3	142.6	22
18	139.4	9	17.4	12.5	133.2	8
19	139.2	22	2.5	2.4	135.9	18
38	142.7	14	8.2	12.2	137.5	12
39	137.9	40	7.3	3.5	136.9	42
11	142.8	16	12.1	22.0	149.9	18
13	124.3	20	0.6	3.5	134.8	19
14	146.5	24	4.5	8.4	148.7	24
15	133.8	24	5.4	5.9	129.8	32
16	129.3	10	12.3	12.8	133.4	10
Mean**			5.5	6.7***		
+1SD			14.8	14.2		
-1SD			2.0	3.1		

*Hemoglobin (Hb), ferritin and iron (Fe) absorption from a diet of corn masa tortillas and refried black beans fortified with ferrous fumarate without added Na_2EDTA (A) and with added Na_2EDTA at a molar ratio 1:1 (relative to total Fe in the fortified corn masa flour) (B).
**Geometric mean
***NS (p=0.5)

Table 5.
Bioavailability Study 2*

ID	Hb (g/L) (A)	Ferritin (μg/L) (A)	Fe absorption (%) (A)	Fe absorption (%) (B)	Hb (g/L) (B)	Ferritin (μg/L) (B)
25	148.2	40	8.6	3.7	133.9	42
26	142.2	23	7.1	5.0	142.8	20
27	147.8	21	15.0	23.3	140.6	18
28	137.9	48	2.2	1.9	144.2	40
29	137.5	28	5.2	9.1	133.2	20
40	155.0	30	3.6	3.6	143.4	22
31	143.7	36	4.4	3.5	143.9	34
32	145.7	24	7.3	7.9	145.7	32
33	146.1	10	4.2	3.9	146.5	19
35	141.5	10	12.1	9.8	141.6	20
36	143.4	28	8.6	8.2	143.3	24
Mean**			6.2	5.8***		
+1SD			10.8	11.5		
-1SD			3.6	2.9		

*Hemoglobin (Hb), ferritin and iron (Fe) absorption from a diet of corn masa tortillas and refried black beans fortified with ferrous fumarate without added Na_2EDTA (A) and with added Na_2EDTA at a molar ratio 1:1 (relative to fortification Fe in the fortified corn masa flour) (B).
** Geometric mean
***NS (p=0.5)

Table 6.
Bioavailability Study 3*

ID	Hb (g/L) (A)	Ferritin (μg/L) (A)	Fe absorption (%) (A)	Fe absorption (%) (B)	Hb (g/L) (B)	Ferritin (μg/L) (B)
21	144.3	21	17.3	18.2	144.9	16
22	149.5	14	3.7	12.4	149.0	16
23	138.6	24	17.4	25.6	138.6	14
24	139.0	23	2.8	2.2	139.0	22
37	144.8	30	1.8	3.4	145.2	24
01	140.4	5	13.8	24.2	152.4	5
02	147.8	18	8.4	18.8	142.5	18
03	125.3	48	0.6	1.6	140.5	28
04	136.1	26	7.1	6.0	152.2	24
12	150.3	10	9.4	18.2	136.6	10
30	150.0	34	5.0	5.6	140.5	32
Mean**			5.5	9.0***		
+1SD			16.5	25.5		
-1SD			1.8	3.2		

*Hemoglobin (Hb), ferritin and iron (Fe) absorption from a diet of corn masa tortillas and refried black beans fortified with ferrous sulfate (A) and with NaFeEDTA (B).
** Geometric mean
*** NS (p=0.5)

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IV. SUMMARY AND CONCLUSIONS

Summary

Key findings from the fat oxidation studies
Results from the hexanal and pentane studies reveal that all iron fortified samples of corn masa flour behaved similarly to the control sample, which contained no added iron. All samples were stable for up to 2 months storage at 65° C and all oxidized after 3 months storage. There is no evidence that any of the added iron compounds will influence fat oxidation or reduce shelf life of stored corn masa flour.

Key findings from the sensory difference evaluation:
Elemental iron emerged as the best iron compound based on the triangle test results. Panelists reported that they could not detect any difference in tortillas made with elemental iron compared to the control (no added iron). Ferrous sulfate did not produce a difference in tortillas made with freshly fortified flour (Phase I), but differences with regard to color, taste, and texture properties detected made after the flour's two months storage (phase II). Differences were also detected in tortillas made from ferrous fumarate in the phase II sensory testing. Furthermore, consumers detected differences in tortillas fortified with NaFeEDTA and ferrous fumarate plus Na_2EDTA, suggesting possible negative sensory characteristics.

Key findings from iron bioavailability studies:
NaFeEDTA performed the best among the iron compounds tested in this study. The MEAN absorption of iron from added NaFeEDTA (9.0%) was significantly higher than from ferrous sulfate (5.5%). The fractional absorption of ferrous fumarate (5.5%, Study 1, and 6.2%, Study 2) and ferrous sulfate (5.5%) was better than anticipated given the high phytic acid content of the test meal. No statistically significant difference in iron absorption was observed when ferrous fumarate was tested with/without added Na_2EDTA. Elemental iron was not evaluated in this study.

Conclusions

Ferrous fumarate
Ferrous fumarate emerged as the compound with the best overall performance of all the iron compounds evaluated in corn masa flour based on storage, sensory, and bioavailability characteristics. During the bioavailability study, ferrous fumarate performed well, with a fractional absorption similar to that of ferrous sulfate. Ferrous fumarate also did well in the sensory studies when added to fresh corn masa flour. However, consumers did note a difference in tortillas made from stored flour containing ferrous fumarate. From the storage perspective, results from the fat oxidation studies showed that ferrous fumarate and the other iron compounds behaved similarly—all were stable after two months storage and all oxidized after three months. The storage characteristic, therefore, does not appear to be a major determining factor in selecting the most suitable compound for fortification of corn masa flour.

NaFeEDTA
Judging by bioavailability study results alone, NaFeEDTA performed the best. It was shown to increase iron absorption of the test meal considerably, as compared to the standard, ferrous sulfate. Its impact on sensory characteristics is a potential drawback as results indicated possible unacceptable color changes in tortillas made with NaFeEDTA

Elemental Iron
Elemental iron outperformed the other iron compounds in the sensory evaluation. There was no detectable difference in freshly fortified tortillas or ones made with stored corn masa flour compared to the control. Fortification with elemental iron is attractive because of its relatively low cost and its demonstrated stability in the food product. However, recommending elemental iron as a fortificant is problematic given that the bioavailability of elemental iron used in this study is not known. It was not evaluated due to the difficulty in preparing labeled elemental Fe that is identical to the commercial products. Previous reports indicate that its bioavailability is variable and at best, about half that of ferrous sulfate. Theoretically, it is possible to suggest the use of elemental iron at 2-3 times the fortification levels used in this study (e.g. 10-mg Fe/100g corn masa flour) but because of the uncertainty over its absorption, there is a possibility that it will not be adequately absorbed. There is also a lack of data on the absorption of different types of elemental iron (electrolytic, carbonyl, reduced), making it unclear which type to use for fortification. To estimate elemental iron's efficacy is a challenge given that isotopic absorption studies of elemental iron have reported conflicting results. Without demonstrated efficacy, it is hard to evaluate elemental iron's impact on improving iron status, the ultimate goal of iron fortification programs.

As for the other compounds tested, it is noteworthy that Na_2 EDTA had no enhancing effect on absorption from ferrous fumarate. Ferrous sulfate performed well in the bioavailability study but the consumer reaction after two months storage raises questions about its sensory characteristics. Results from the sensory study suggest negative changes in flour fortified with ferrous fumarate plus Na_2EDTA.

In sum, based on the combined results from the storage, sensory and bioavailability tests, ferrous fumarate appears to be the most promising iron compound for corn masa fortification. To further examine the constraints related to the use of elemental iron and NaFeEDTA and thus better assess the potential of these iron compounds as corn masa flour fortificants, additional research may be needed. These could include more sensory tests on ferrous fumarate and NaFeEDTA to evaluate the difference detected in tortillas fortified with these compounds. Another aspect to take into consideration is the varying prices of the iron compounds. The bioavailability of elemental iron is a critical factor that will determine its usefulness as an iron fortificant for corn masa flour.

APPENDIX 1

Corn Masa Flour Advisory Panel Members

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