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Study of the effect of Ca, Fe and Zn content on the recovery of phytic acid in infant cereals with the HPLC-RI Method. Problems and solutions

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Abstract

Phytic acid is an antinutrient that inhibits metal/mineral absorption, affecting the nutrition of infants. The high performance liquid chromatography with refractive index (HPLC-RI) has been the commonly used technique for the determination of phytic acid (IP6) in infant cereals. Despite numerous studies, the low phytic recovery still affecting the accuracy of the determination. In this paper, a HPLC-RI methodology was optimized focusing on the factors that may affect the accuracy of the method. The results indicate that infant cereals, usually having high mineral content, present recoveries in a range from 48 to 57% that significant contrast with the approximately 92% recoveries obtained with grains wheat whose mineral content is low. It seems that insufficient recoveries are produced by the precipitation of the phytic acid during the extraction process, induced by the mineral content in the infant cereal. The efficiency of recovery of phytic acid in presence Fe and Ca was improved by the use of trichloroacetic acid. This recovery depends of concentration of phytic acid in the sample. Zn was not considered in this study because all the cereals under study presented similar concentration.

Key words: Phytic acid, infant cereal, high performance liquid chromatography, recovery and trichloroacetic acid.

Estudio del efecto del contenido de Ca, Fe y Zn en la recuperación del ácido fítico en cereales infantiles con el Método HPLC-RI. Problemas y soluciones

Resumen

El acido fítico es un antinutriente que inhibe la absorción metal/mineral afectando la nutrición de los infantes. La cromatografía liquida de alta eficiencia con detección por

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índice de refracción es la técnica más comúnmente usada para la determinación de ácido fítico en cereales infantiles. Sin embargo, después de numerosos estudios algunos problemas siguen presentes. Especialmente las bajas recuperaciones reportadas por algunos autores. En este trabajo se optimizó una metodología HPLC-RI enfocada en mejorar los factores que afectan la exactitud del método. Se observa que para cereales infantiles, generalmente con alta concentración de minerales, se obtiene recuperaciones entre 48 y 57 % en contraste al 92% obtenido en semillas de trigo cuyo contenido mineral es bajo. Estas bajas recuperaciones son producto de la precipitación del ácido fítico durante la extracción, generada por el alto contenido mineral de los cereales infantiles. La eficiencia de recuperación del ácido fítico en presencia de Fe y Ca se incrementó con el uso de ácido tricloroacético. Esta recuperación depende de la concentración de ácido fítico en la muestra. El Zn no fue considerado en este estudio debido a que los cereales evaluados presentaron similar concentración.

Palabras clave: acido fítico, cereales infantiles, cromatografía liquida de alta eficiencia, recuperación, ácido tricloroacético.

Introduction

Phytic acid is a natural constituent in grains, cereals and legumes. Throughout the years its anti-nutritional behavior has been studied because it can bind essential minerals, such as Ca, Fe and Zn, causing a deficiency of these minerals in humans, particularly in high risk groups such as infants, children, pregnant women and elderly people (1). The high performance liquid chromatography with refractive index detector (HPLC-RI) has been extensively used in the study of the phytic acid in unprocessed and infant cereals (1-15) however; authors such as Books and Lampi (3) and Park et al. (4) have stated that the HPLC-RI method generates very low recoveries in infant cereals. Books and Lampi (3) have suggested that such low recovery percentages could be related to the high mineral content of these cereals. On their part, Lehrfeld (2) and Amaro et al. (9) have reported problems associated with the HPLC-RI method and provide some corrections and solutions. The above authors do not explain the origin of the low recovery of the phytic acid, which can be produced by: the high mineral content of the samples that could affect the treatment of the same and/or the use of non-optimized methodologies that could lead to poor recoveries in the analysis of infant cereals. This work focused on the understanding the causes of low phytic acid recoveries when HPLC-RI technique was used for the analysis of infant cereals. To reach our goal, it was studied the interferences of metal such as Ca and Fe together with the use of trichloroacetic acid as alternative to reduce such interferences. The samples were infant and unprocessed cereals. Phytic acid standards, with and without metal addition, were also included for comparison purposes.

Materials and Methods

Materials and reagents

The samples were prepared with phytic acid-di-potassium salt 95% w/w provided by Aldrich Chemical Company (Milwaukee, WI, USA). The purity of this compound was determined by elemental phosphorus analysis by ICP-OES. Stock standard solutions of Ca, Fe, Zn, hyper pure nitric acid and 90% w/w purity formic acid were supplied by BDH Laboratory Reagents Company (Poole, England). Potassium chloride salt, 99.99% w/w, tetraethylammonium hydroxide (TENOH, 40 % w/w in aqueous solution), trace select ultra HCl 30% and ACS reagent trichloroacetic acid (TCA) 99.0% w/w were supplied by Aldrich Chemical Co (Milwaukee, WI, USA). NH2 sep-pack cartridge was supplied by Millipore Corporation (Milford, Mass, USA). Ion exchange resin (AG 50W-X4 of H^+, 50-100 mesh) was provided by Bio-Rad Company (Richmond, CA, USA). 99.8% w/w high purity methanol was delivered from the Riedel-de Haën AG Company (Seelze, Hannover, Germany). 18 MΩ/cm deionized water was used through this work.

Instrumentation

Flame Atomic Absorption Spectrophotometry (FAAS)

The Ca, Fe and Zn content were analyzed by flame atomic absorption spectrophotometer GBC Avanta with a deuterium arc lamp to background correction (GBC Scientific Equipment, Dandenong, Australia).

HPLC System

The HPLC System consisted of a Hewlett Packard 1100 (now Agilent Technologies, Santa Clara, CA) solvent delivery system, a Rheodyne 9125 six-port injection valve (Cotati, USA) with a 20 μL PEEK injection loop, a Hamilton PRP-1 PEEK column (5 μm, 150 mm length, 4.1 mm i.d) (Reno, NV, USA) and a refractive index detector model 1047A, Hewlett Packard (Santa Clara, CA, USA). The analog output from the detector was connected the A/D converter model SS420 and the data were processed with EZCHROM software, provided by the Scientific Software, Inc. (Pleasanton, CA, USA). Connections between injection valves, injection loop, pump and chromatographic column were accomplished with 0.250 mm internal diameter, PEEK material pipe. Solvent was pumped at a flow rate of 1 mL/min. The mobile phase was a 0.015 mol/L formic acid solution having 5% w/w methanol and 0.14% w/w tetraethylammonium hydroxide. The column and detector temperature were kept at 30°C.

Sample collections and preparation

The infant cereals were collected in July 2007 in different local market in the City of Caracas, Venezuela and the wheat was supplied in January 2008 by the Molinos Nacionales Industry in Puerto Cabello, Venezuela.

All samples were freeze-dried (Freezone 6, Labconco) to constant weight. Dried food samples were packaged in plastic and storage at room temperature until analyzed in March 2008.

Analysis

FAAS analysis

Approximately 5 g freeze dried food samples (four replicates) were ashed in a muffle furnace for 24 h at 450ºC. Then, a few drops of hyperpure nitric acid were added, and the sample re-ashed at 450ºC for an additional 24 h. After cooling, the treated samples were dissolved in 6 mol/L trace select ultra HCl (8 mL) and diluted to 100 mL with distilled deionized water. Concentrations of Ca, Fe and Zn were determined in the aliquots using a flame atomic absorption spectrophotometer. For Ca determinations, nitrous oxide-acetylene flame and a potassium ionization buffer were used.

HPLC-RI analysis

Extraction (a).- Phytic Acid and other inositol phosphates were extracted from approximately 1 g of each freeze-dried sample with 10 mL of 0.5 mol/L HCl. The tip of the ultrasonic microprobe was inserted halfway into the solution, and the sample was sonicated for 1.5 min (40% output and 25 watts) at room temperature. The suspension was centrifuged at 3400 rpm for 15 min at room temperature. The supernatant was decanted into a 25 mL volumetric flask and the residue was washed with 5 mL of 0.5 mol/L HCl. This second supernatant was centrifuged and combined with first supernatant and volume made up to 25 mL with water.

Purification (b).- An aliquot (10 mL) of supernatant was diluted with 50 mL of H_2O and poured onto a NH2 sep-pak of 500 mg, aided by the use of a disposable syringe. The loaded NH2 sep-pak was washed with 10 mL of a 0.05 mol/L HCl solution and the inositol polyphosphates were then eluted with 3 mL of a 2 mol/L HCl solution into 50 mL recovery flasks. Eluted samples were dried by vortex evaporation in a vacuum at 40 ºC. The residue was re-suspended with 2 mL mobile phase solution and centrifuged at 3400 rpm for 5 min at room temperature. 20 µL of the clear supernatant was injected into the HPLC unit.

Colorimetric analysis

The standard (10 mL aliquot) was purified following the procedure purification **b** of the HPLC-RI analysis section. The residue was re-suspended with 10 mL distilled water. Three-milliliter of the samples was pipetted into 15-mL conical centrifuge tubes with 3 mL of water. 1 mL of the modified Wade reagent (0.03% $FeCl_3.6H_2O$ and 0.3% sulfosalicylic acid in distilled water) was added to each tube. Finally the solution was mixed on a vortex mixer for 5 s and centrifuged for 10 min. The absorbance of the supernatant was taken at 500 nm using water to fix the zero. The individual absorbances were measured on a Genesys 10.

Recovery

The spiking method was carried out in order to evaluate the recovery. It was used phytic acid potassium solutions at concentration level of 100 mg/L before extraction methods.

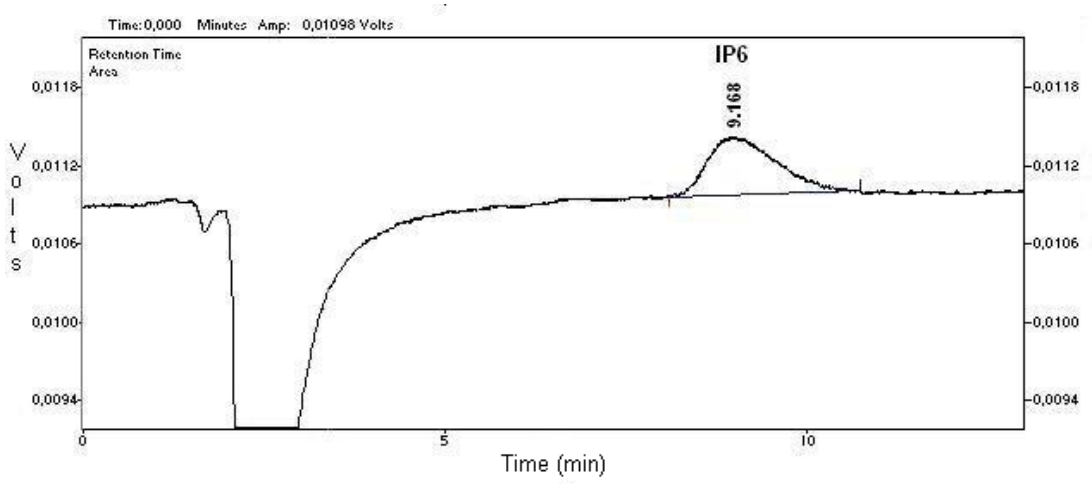
Statistical analysis

The software STATGRAPHICS CENTURION (Statpoint Technologies Inc., 2012) was used for regression

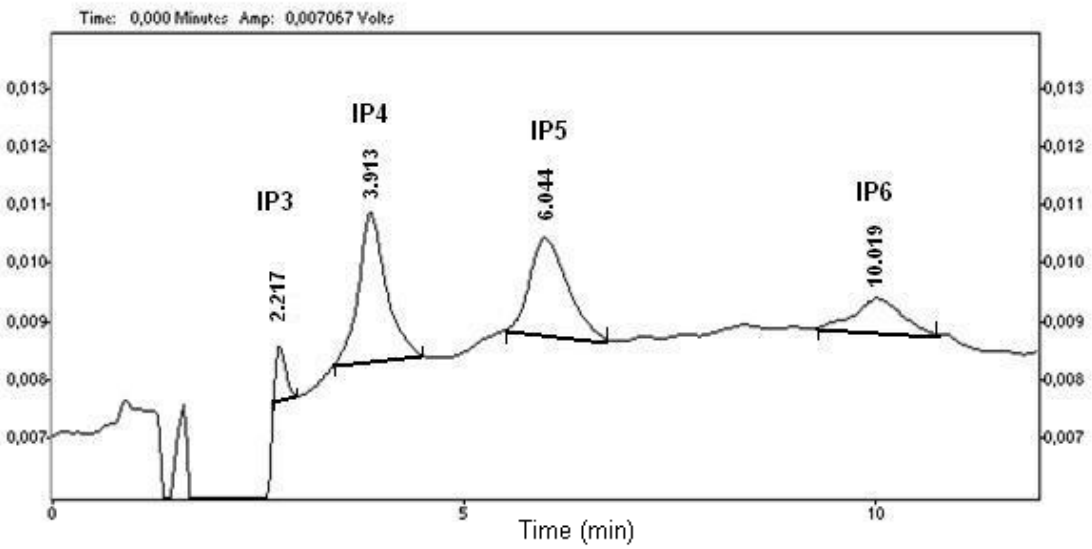
analysis. The results were expressed as the mean ± standard considered significantly different at $p \leq 0.05$.

Results and Discussion

Figure 1 shows the chromatograms of (a) phytic acid and (b) hydrolyzate of phytic acid (95 ºC, 10 h) (9). It can be appreciated that the IP6 is the peek having the greatest time of retention (Figure 1.a). The chromatography conditions readily resolves IP3, IP4, IP5 and IP6 (Figure 1.b).



(a)



(b)

Figure 1. Chromatograms of **(a)** phytic acid and **(b)** hydrolyzate of phytic acid, hydrolysis at 95 ºC for 10 h. Chromatography conditions: column type PRP-1 Hamilton, 150x4.6 mm i.d, 5 µm particle size. Mobile phase: 0.015 mol/L formic acid, 5%, w/w, methanol and 0.14%, w/w, tetraethylammonium hydroxide; the elution rate was 1 mL/min.

Metals Contents and Recovery

Table 1 (10) summarizes the content of Ca, Fe, Zn and the phytic acid recovery for three infant cereals and wheat. It can be noticed that recovery of the phytic acid is relatively low (52 – 57%) in infant cereals with a high mineral content (Ca: 357-440, Fe: 10.6-14.8, and Zn: 0.73-1.12 mg/100g) when compared with 92% found with a low mineral content (Ca: 35.2, Fe: 2.12, and Zn: 2.2 mg/100g). These results clearly showed that low recovery of the phytic acid in infant cereals was related to the high metal content of the studied samples.

Table 1. Content of Fe, Ca, Zn, phytic acid and recovery of phytic acid in the evaluated samples

Sample	***Iron mg/100g***	***Calcium mg/100g***	***Zinc mg/100g***	***Phytic acid % w/w***	***Phytic acid Recovery %***
Wheat	2.12 ± 0.05	35.2 ± 0.6	2.2 ± 0.1	0.92 ± 0.02	92 ± 4
Wheat cereal	10.6 ± 0.6	440 ± 8	0.73 ± 0.01	0.059 ± 0.001	52 ± 6
Rice cereal	14.1 ± 0.8	344 ± 10	1.12 ± 0.03	0.087 ± 0.008	54 ± 2
Corn cereal	14.8 ± 0.6	357 ± 14	0.38 ± 0.01	ND	56.5 ± 0.7

ND, not detection

Effects of Metals in the Recovery

A number of experiments were performed to verify whether the low recovery of phytic acid was caused by the high metal content in infant cereals. For this reason, several standard phytic acid solutions with and without metals were prepared and the percentage recovery of phytic acid determined with the described method. Ca and Fe were used at concentrations similar to that found in the infant cereals. Zn was not included in this study because all the samples under study had similar content.

Standard 1: contain phytic acid (120 mg/L) in HCl 0.5 mol/L; standard 2: with phytic acid (120 mg/L), Ca (140 mg/L) and Fe (6 mg/L) in HCl 0.5 mol/L; standard 3: with phytic acid (240 mg/L) in HCl 0.5 mol/L and standard 4: with phytic acid (240 mg/L), Ca (140 mg/L) and Fe (6 mg/L) in HCl 0.5 mol/L. In this study, samples of 10 mL standard solutions were taken 20 minutes after being prepared, purified and injected to the chromatograph as indicated in the procedure purification **b** of the HPLC-RI analysis.

The arithmetic mean of recoveries for phytic acid were: standard 1; 104 ± 2%, standard 2; 60 ± 5 %, standard 3; 100 ± 2 % and standard 4; 81 ± 3 %. It was noticeable that standards 2 and 4 (with metals) had lower recoveries when compared with standards 1 and 3 (without metals). This suggested that the phytic/metal ratio was important in the recovery efficiency of phytic acid.

Effect of type of acid, acid concentration and digestion time on the recovery of phytic acid

It has been determined that the presence of metals such as Ca and Fe, influence the recovery of phytic acid in infant cereals; however, its effect was only reported by some researchers. Brooks and Lampi (3) reported high concentrations of phytic acid in infant cereals with the HPLC method due to ionic exchange (UV-Vis detection), while through the ionic pair method (RI detection), concentrations are substantially lower.

Likewise, Park et al. (4) indicated a better recovery with the AOAC method 986.11 (15) in comparison to the liquid chromatographic ionic pair and GC-FID methods. Nevertheless the AOAC method overestimates the phytic acid content in processed foods because it did not differentiate phytic acid from partially dephosphorylated phytic acids such as IP5 and IP4 (16). Some authors have evaluated the effect of the phytic acid on the availability of certain metals in cereals and legumes (14, 18-22) and reported the feasibility to find phytic acid/metal insoluble compounds. It is possible that the sample treatment may prevent the formation of the phytic acid/metal compounds and generates higher recoveries of phytic acid using HPLC-RI method. Going over the sample treatments (1-15), it was noticeable the use of different acids, reactive concentrations and times of extraction in the methodologies and maybe these facts, were the source of inconsistencies in the recovery factors. It this study we assessed the effect of type of acids, acid concentration and digestion time on the recovery of phytic acid in the presence of metals. For this purpose 120 and 240 mg/L phytic acid standards in 0.5 and 1.0 mol/L HCl and 0.6 and 0.9 mol/L TCA were prepared. All of these standards contain 140 mg/L of Ca and 6 mg/L of Fe. Samples of 10 mL were taken 20 and 150 min after being prepared. Once the aliquot of sample was taken, it was subject to the purification process and injected to the chromatograph as indicated in section **b** of the HPLC-RI analysis. Room temperature was used in all the experiments.

It was explored the effect of some factor on the recovery. For this purpose, experimental design of $\mathbf{2^3}$ was used and the following independent variables were selected: A (digestion time), B (acid concentration) and (C acid type) with two levels of variation (-1 and +1). This design was individually applied

to each concentration of phytic acid evaluated.

Table 2 shows a summary of the percentages of recovery of phytic acid from all of the experiments performed for the two concentrations evaluated. The tests 1 to 8 are replicated as tests 9 to 16.

Table 2. Planning assay 16 matrix with independent variables, experimental responses Y for recovery % of 120 and 240 mg/L phytic acid

Assays	Code (real)			Recovery, % Y	
	Digestion time, min	Acid concentration, mol/L	Acid type		
	A	B	C	120 mg/L	240 mg/L
1	-1 (20)	-1 (0.5)	-1 (HCl)	47	73
2	+1 (150)	-1 (0.5)	-1 (HCl)	54	77
3	-1 (20)	+1 (1.0)	-1 (HCl)	50	80
4	+1 (150)	+1 (1.0)	-1 (HCl)	49	70
5	-1 (20)	-1 (0.6)	+1 (TCA)	40	90
6	+1 (150)	-1 (0.6)	+1 (TCA)	50	70
7	-1 (20)	+1 (0.9)	+1 (TCA)	40	86
8	+1 (150)	+1 (0.9)	+1 (TCA)	63	74
9	-1 (20)	-1 (0.5)	-1 (HCl)	52	70
10	+1 (150)	-1 (0.5)	-1 (HCl)	52	75
11	-1 (20)	+1 (1.0)	-1 (HCl)	56	78
12	+1 (150)	+1 (1.0)	-1 (HCl)	54	72
13	-1 (20)	-1 (0.6)	+1 (TCA)	46	88
14	+1 (150)	-1 (0.6)	+1 (TCA)	54	72
15	-1 (20)	+1 (0.9)	+1 (TCA)	44	85
16	+1 (150)	+1 (0.9)	+1 (TCA)	60	75

Tables 3 and 4 present the effects of the variables for recovery of 120 mg/L and 240 mg/L of phytic acid, respectively. According to the regression coefficients for 120 mg/L of phytic acid (Table 3), the variable A (digestion time) and the interaction AB, had a significant effect on the response (Y), the variables B (acid concentration) and C (acid type) and the interactions AB and BC showed no significant effects.

The highest level of phytic acid recovery was obtained under the following conditions: 150 min digestion time (A = +1) and 0.9 mol/L of TCA (B = +1) and (C = +1) for tests 8 and 16 (Table 5) with 60 %. In comparison with tests 2

and 10, the most extensively used condition in the bibliography; a 10% increase in the recovery is obtained.

The results for 240 mg/L of phytic acid (Table 4) showed that the variables A (digestion time) and C (acid type) and the interaction AC, had a significant effect on the response. The sign of the C variable was positive, while the signs of the variable A and the AC interaction ware negative, and the greatest response was obtained at a low time of digestion and TCA.

This was contrary to the previous case where the greatest response took longer times. The highest level of phytic acid recovery was achieved under the following conditions: 20 min digestion time (A = -1) and 0.6 mol/L of TCA (B = -1) and (C = +1) for tests 5 and 13 (Table 4) with a recovery of 90 %. This was a 20% increase in recovery compared to test 2 and 10.

Table 3. Regression coefficients for response Y = recovery % of 120 mg/L phytic acid

Variation source	Regression coefficients	Standard error	F-statistics	p-value
Mean	50.8125	0.986233		
A: Digestion time	**7.875**	**1.97247**	**15.94**	**0.0040***
B: Acid concentration	2.375	1.97247	1.45	0.2630
C: Acid type	-2.375	1.97247	1.45	0.2630
AB	1.125	1.97247	0.33	0.5841
AC	**6.375**	**1.97247**	**10.45**	**0.0120***
BC	1.875	1.97247	0.90	0.3696
Block	2.875	1.97247	2.12	0.1831

* Significant effect

Table 4**.** Regression coefficients for response Y = recovery % of 240 mg/L phytic acid

Variation source	Regression coefficients	Standard error	F-statistics	p-value
Mean	77.1875	0.922801		
A: Digestion time	**-8.125**	**1.8456**	**19.38**	**0.0023***
B: Acid concentration	0.625	1.8456	0.11	0.7436
C: Acid type	**5.625**	**1.8456**	**9.29**	**0.0159***
AB	-1.375	1.8456	0.56	0.4776
AC	**-6.375**	**1.8456**	**11.93**	**0.0086***
BC	-0.625	1.8456	0.11	0.7436
Block	-0.625	1.8456	0.11	0.7436

* Significant effect

The contrasting effects presented at low and high phytic acid concentration was difficult to explain. However, the results of Liang et al. (20) can help to clarify this tendency. They found different solubilities of Ca at different [IP6]/[Ca] ratios. Likewise, the effects can be attributed to the substantial differences in the solubility properties at low ([IP6]/[metal] < 1) and high ([IP6]/[metal] > 1) phytic acid ratios respect to the metal concentrations. Nevertheless, it was not considered the shaking procedure in the study. This factor may have an influence in the dissolution of the real samples and should be considered in further experiments.

It was tested the recovery factor of the phytic acid in presence of some metals to evaluate the effect of HCl and TCA. It was used the colorimetric method proposed by Latta and Eskin (23). The mean average for standard A (120 mg/L IP6, with metals and 0.5 M HCl), B (240 mg/L IP6, with metals and 0.5 M HCl), C (120 mg/L IP6, with metals and 0.6 M TCA) and D (240 mg/L IP6, with metals and 0.6 M TCA were 38 ± 1; 73 ± 3; 68 ± 2 and 90 ± 1, respectively. The statistical test to compare means showed evidence that there were statistical significant differences among the standards demonstrating that trichloroacetic acid improved the recover of the phytic acid.

The above results are in agreement with those obtained by Brooks and Lampi (3), they reported higher IP6 concentrations using 0.6 mol/L TCA in the extraction process in the analysis of infant cereals by anionic exchange-HPLC (UV-Vis detection), but this technique use post-column detection for the analysis of phytic acid.

Conclusions

The results show that the low recovery of phytic acid in infant cereals through the HPLC-RI is due to the high content of minerals in these samples, which produces the precipitation of phytate during the extraction process. With the use of the TCA, the percentage recovery be can increased by 10 to 20% depending on the concentration of phytic acid in the infant cereal. The conditions of concentration of the TCA and digestion time depend on the concentration of phytic acid in the cereal. For low concentrations, long times and for high concentrations shorter times of digestion are required.

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