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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · FEBRUARY 2002

Impact Factor: 2.91 · DOI: 10.1021/jf010792w · Source: PubMed

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Simultaneous and Sensitive Analysis of Cu, Ni, Zn, Co, Mn, and Fe in Food and Biological Samples by Ion Chromatography

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A sensitive and simple method for the simultaneous determination of nutritionally important minerals in food samples is in great demand. Ion chromatography coupled with UV−vis detection is shown to be an appropriate technique for this objective. The method is based on the formation of mineral complexes by pyridine-2,6-dicarboxylic acid in the mobile phase. The complexes are then postcolumn derivatized with 4-(2-pyridylazo)resorcinol (PAR), resulting in mineral−PAR complexes that are detected by UV−vis absorption at 500 nm. This facilitates the simultaneous separation and quantification of minerals in one chromatographic run. Within 16 min, Cu, Ni, Zn, Co, Mn, and Fe are analyzed. When a 50 μL injection volume is used, the average detection limit is 5 ppb in the injection liquid. The detection limit makes it a superior alternative to AAS and, in several applications, also an alternative to ICP-MS techniques. Different sample treatments were evaluated. The concentration of acid in the treated sample varied with the sample treatment, which may cause a limitation for the injection volume. A crucial prerequisite to achieve the reported detection limits and to obtain reliable results is to completely exclude all contamination from instruments and materials.

KEYWORDS: Minerals; complex; ion chromatography; UV−vis absorption

INTRODUCTION

Simultaneous analysis of different minerals in food or biological samples is a frequent request. At the present time the most common methods for mineral analyses are atomic absorption spectroscopy (AAS) or inductively coupled plasma−mass spectroscopy (ICP-MS). AAS is time-consuming because usually only one mineral is analyzed at a time; another disadvantage is that relatively large sample volumes are used. Besides, for some samples a lower detection limit is necessary than can be achieved by AAS. ICP-MS is an excellent analysis method, but expensive. Both ICP-MS and ion chromatography (IC) require relatively experienced laboratory personnel for implementation and maintenance. The IC system can with minor modifications be used in analysis for the determination of other ionic components (Fredrikson et al., unpublished data; *1*).

The presented IC method was developed to simultaneously analyze the transition metals Cu, Ni, Zn, Co, Mn, and Fe with low detection limits, at low cost and with easy handling. The possibility of using small sample volumes was also desired. The method is basically an application of the system by Dionex (*2*−*7*). A crucial concern when the method was set up was to exclude all mineral contamination from the instruments and materials. Materials in all instrument details were therefore rigorously controlled and changed to customized details when necessary. Materials for the sample preparation procedures were also evaluated with respect to contamination.

The separation of heavy and transition metals with ion exchangers requires complexation of the metal ions in the mobile phase to reduce their effective charge density (*8*). Because the selectivity coefficients for transition metals of the same charge hardly differ, a selectivity change is obtained by the introduction of a secondary equilibrium, a complexation equilibrium, which is obtained by adding suitable complexing agents to the mobile phase (*8*). In the present method the complexing agent used in the mobile phase is pyridine-2,6-dicarboxylic acid (PDCA). This compound forms anionic or neutral complexes with the metal ions, which are separated by the column. For detection a postcolumn reaction with 4-(2-pyridylazo)resorcinol (PAR) is used, which can be detected by UV−vis absorbance at 500 nm.

Alternative combinations of separation and detection methods are possible. A mobile phase with mandelic acid, oxalic acid, or tartaric acid as complexing agents is reported (*8*, *9*). Furthermore, separation by polybutadiene maleic acid, C_{18}, or unmodified silica gel columns is possible, and direct conductivity is reported to function as a detection method (*8*, *10*, *11*). The method presented in this work was considered to be promising for the analytes of interest.

Similar analyses of transition metals by IC have been done previously (*12*−*14*), and mostly the method is applied for analyses of transition metals in simple matrices, for example, in water or similar sample types. However, in the present work

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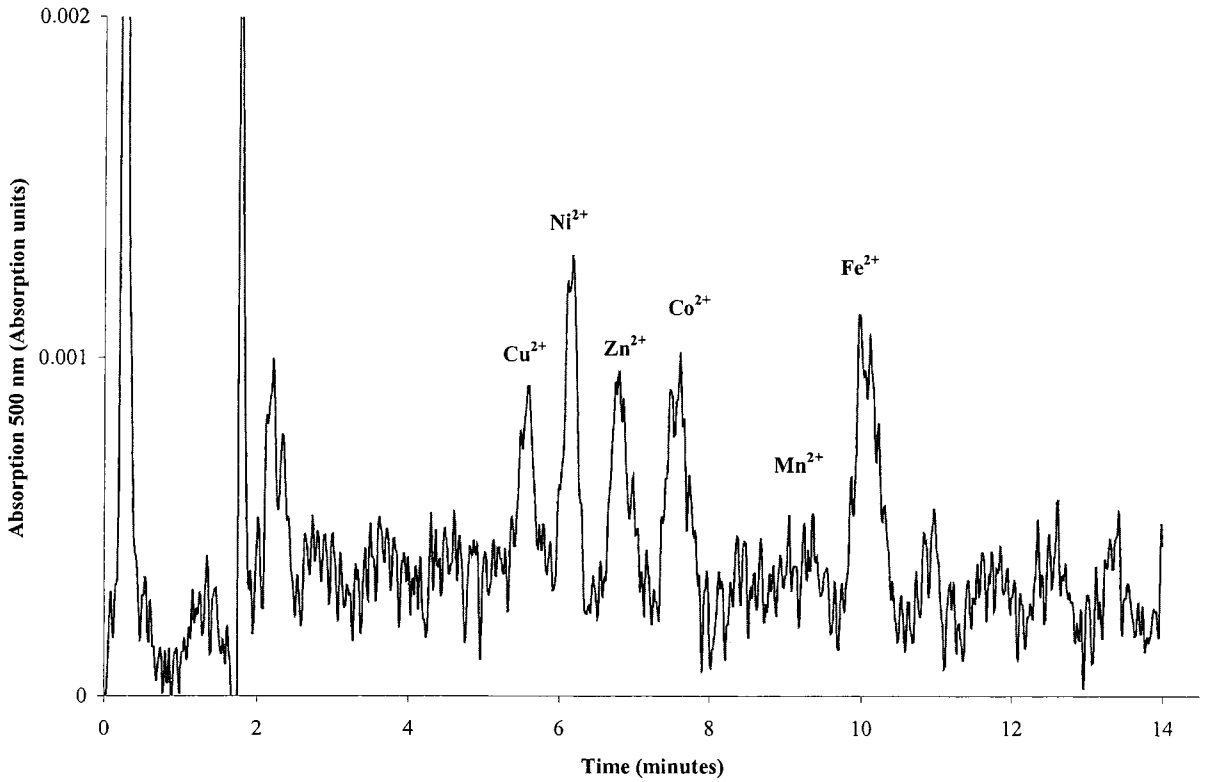

Figure 1. Chromatogram from 25 μL injection of 10 ppb standard solution. The average detection limit for the six minerals corresponds to ~5 ppb with 50 μL injection.

the method is applied to food and biological samples, which implies special difficulties regarding the sample matrix that rarely are reported by previous publications, with the exception of Dionex (*3*, *7*) and Buldini et al. (*1*).

Other alternative IC methods for transition metal analyses have been published previously (*15*−*18*).

MATERIALS AND METHODS

Reagents. PDCA, PAR, and transition metal standard solutions of 1000 ppm concentrations for IC and AAS were purchased from Fluka (Buchs, Switzerland). Deionized water was purified by a Millipore (Bedford, MA) water system to a specific resistance of 18 MΩ·cm or greater. All other reagents used were of analytical grade and obtained from commercial sources.

The mobile phase was composed of 1 g of PDCA, 20 g of sodium acetate ($C_2H_3NaO_2 \times 3H_2O$), 8.5 g of acetic acid ($C_2H_4O_2$), and 0.2 g of ascorbic acid in 1 L of H_2O, pH 4.3 ± 0.1. The ascorbic acid was added to prevent an observed oxidation of the mobile phase. Despite this, we recommend fresh solutions. The postcolumn reaction solution was composed of 240 mL of 25% ammonium ($NH_{3(aq)}$), 77 mg of PAR, and 57 mL of acetic acid diluted to 1 L, pH 10.2 ± 0.1. The pH of the PAR solution determines which metals that can be detected. The PAR postcolumn reagent solution was continuously degassed with helium, because PAR is easily oxidized by oxygen. The PDCA eluent flow was kept at 0.8 mL/min, and the PAR postcolumn flow was 0.4 mL/min. This combination showed the optimal absorption. The standard solutions were prepared to contain 0.05 M HCl and ascorbic acid 2 g/L.

Instrumentation. The IC system consisted of a Waters 626 gradient pump PEEK (polyether ether ketone) (Milford, MA), a Triathlon PEEK autoinjector, and plastic vials by Spark (Emmen, Holland). The autoinjector was customized with an air/prepuncturing needle in titan, a vial sensor strip in PEEK, and a plastic washing chamber. Guard column CS5G and analytical column CS5A were from Dionex Corp. (Sunnyvale, CA). The postcolumn system consisted of a reaction-mixing tee in Teflon (Jour Research, Onsala, Sweden) and a self-made reaction coil consisting of a Teflon tube (i.d. = 0.2 mm) coiled to optimize high blending rate and complex binding, and the avoidance of peak broadening. A postcolumn HPLC pump K-500 in PEEK (Knauer, Berlin, Germany) and a Waters 2487 dual absorbance detector were used. The flow cell in the detector was made of titan. Millenium chromatography software was used. Milestone microwave laboratory system Ethos Plus and a laboratory terminal 800 controller (Sorisole, Italy) equipped with MPR-300/12S (medium-pressure segmented rotor) were used for sample digestion.

For sample treatment plastic materials were used if possible. The glassware, Teflon bombs, and Microcon YM-30 centrifugal filter (Millipore, Bedford, MA) were always acid-washed prior to use, due to observation of contamination. The utensils were stored in closed containers to avoid airborne contamination.

For volume reduction of liquid samples a homemade evaporation equipment was used. An air stream was passed through a 0.22 μm filter and then divided into multiple parallel air streams led into test tubes, to facilitate the simultaneous treatment of multiple samples.

Samples. The liquid samples were dialysates obtained in our laboratory by an in vitro digestion of liquid infant formulas, as described by Fredrikson et al. (*19*). The fermented vegetable mix was prepared in our laboratory. Certified reference materials were "simulated diet A" and "simulated diet E" (*20*), ARC/CL total diet reference material HDP (Agricultural Research Centre of Finland, Jokioinen, Finland), and freeze-dried animal blood (International Atomic Energy Agency, Vienna Austria). Samples of Dona whole maize flour (*Zea mays*), sorghum white flour, and sorghum red flour (*Sorghum vulgare*) were obtained from local market in Dar es Salaam, Tanzania. Infant formulas based on soy (*Glycine max*) or pea (*Pisum sativum*) protein were obtained from Semper AB (Stockholm, Sweden). The feed phytase enzyme "Phytase Novo CT" (granulate) was obtained from Novo Nordisk A/S, Bagsværd, Denmark. The samples were analyzed as duplicates and correspond to dry weight, unless other is stated. The results are reported as average ± standard deviation.

Sample Treatments A—D. Sample treatments were customized to suit different types of samples and mineral matrices.

Treatment A. For analyzing soluble hydrated and weakly complex bound minerals in transparent liquid samples, a simple acidification by addition of HCl was used. The acidification was done by adding

Table 1. Reproducibility

A. Series of Repeated Injections with Alternating Concentrations; Results from Five 50 μL Injections of Each Standard Solution (±SD of Peak Area)

standard solution	Cu	Ni	Co	Zn	Mn	Fe
10 ppb	±14%	±5.2%	±10%	±10%	±21%	±11%
50 ppb	±2.0%	±2.0%	±2.7%	±2.2%	±3.6%	±1.3%
800 ppb	±0.66%	±0.67%	±1.2%	±0.73%	±2.0%	±1.2%

B. Series of Repeated Injections of Microwave-Digested Biological Samples with High and Low Trace Element Contents, Respectively; Results from Five 25 μL Injections of Each Sample (±SD of Peak Area)

sample	Cu	Zn	Mn	Fe
HDP		±2.0%	±4.1%	±1.1%
diet E	±0.9%	±1.0%	±2.7%	±0.3%

0.1 mL of 0.5 M HCl and 0.1 mL of ascorbic acid (20 g/L) to 0.8 mL of sample. The solution was then vortex mixed and centrifuged at 12000*g* for 10 min. If necessary, the supernatant was ultrafiltered through an acid-washed Microcon YM-30 centrifugal filter before injection on the chromatographic system. For analyzing soluble minerals in turbid liquid samples, the acidification can be applied to the supernatant after centrifugation (12000*g* for 10 min). For total mineral analysis in turbid liquids, a leaching or a microwave (MW) digestion procedure is necessary.

Treatment B. For liquid samples with low concentrations of minerals a volume reduction step by evaporation was developed. For example, 8 mL of liquid was reduced to a final volume of 1−1.5 mL by streaming 0.22 μm of filtered air, overnight at room temperature, in test tubes with premarked volume scales. To the reduced sample was added 0.2 mL of 0.5 M HCl and 0.2 mL of ascorbic acid (20 g/L) to a final volume of 2 mL. The solution was vortex mixed and centrifuged prior to injection. The evaporation was not continued to complete dryness due to the difficulties in redissolving the dry residue.

For solid milled dry samples and wet mixed samples, two different sample preparations were used, a leaching treatment and MW digestion.

Treatment C. The leaching was done by dissolving the sample in 2 mL of 2 M HCl and incubating overnight, ~12 h. For dry samples that absorbed large liquid volumes, 4 mL of 1 M HCl was used instead of 2 mL of 2 M HCl. After the incubation, 1 mL of ascorbic acid (20 g/L) and water were added to give a 10 mL total volume. This solution was further incubated during altering sonication and thorough vortex mixing for 2 h. Centrifugation for 10 min at 10000*g* followed, and if necessary the supernatant was also ultrafiltered through a Microcon YM-30 centrifugal filter (Millipore, Bedford, MA). The filtrate was then injected into the IC system. Because of the acid concentration in the leaching-treated samples, maximally 50 μL injection volumes were used.

Treatment D. MW sample digestion was done by mixing maximally 0.4 g of dry sample, 3 mL of H_2O (when using wet samples the water content has to be into consideration), 0.75 mL of concentrated HNO_3 and 0.15 mL of concentrated HCl in a Teflon vial. This exceeds the 2 M HNO_3 minimal limit as described by Walter et al. (*21*) and the use of similar acid content/sample ratio as recommended by Knapp et al. (*22*). The sample was digested to a transparent solution in the MW oven by a continuos temperature program reaching 180 °C in 15 min; this temperature was kept constant for 20 min. After this treatment the sample was cooled to room temperature, and the sample was decanted into a test tube with the 10 mL volume level premarked. The Teflon vial was washed, and the washing water and sample liquid were pooled in the test tube and diluted to a final volume of 10 mL. Before injection, 0.1 mL of ascorbic acid (20 g/L) was added to 0.9 mL of sample. If necessary to reduce the negative consequences of the acid content for the chromatographic performance, the sample was dried and dissolved. It was dried to complete dryness by streaming 0.22 μm of filtered air overnight. To the dried sample were added 0.1 mL of 0.5 M HCl, 0.1 mL of ascorbic acid (20 g/L), and 0.8 mL of H_2O. This solution was sonicated for 15−30 min. The sample was transferred to a 1.7 mL microcentrifuge tube, and finally centrifugation at 12000*g* during 5 min completed the treatment before injection. Normally 50 μL of sample was injected for analysis.

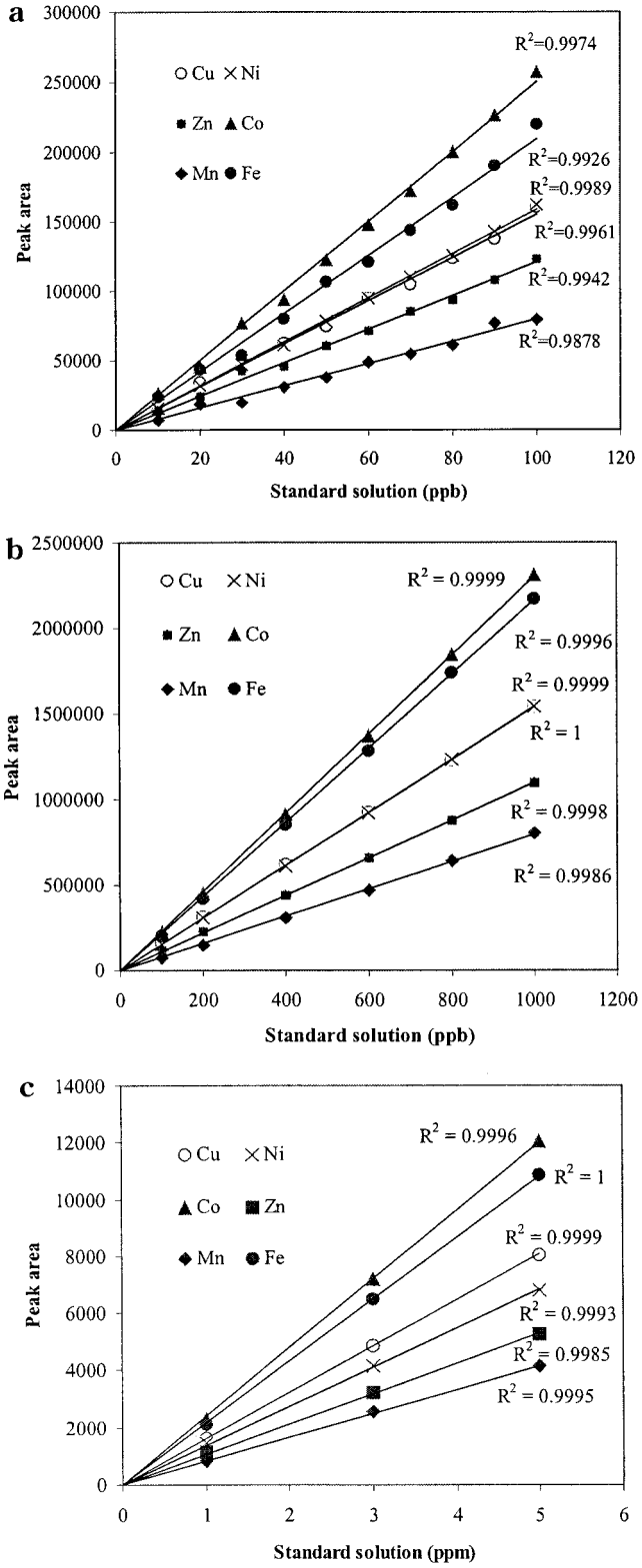

Figure 2. Linearity from single 50 μL injections of standard solutions with Cu, Ni, Zn, Co, Mn, and Fe: (a) range = 10–100 ppb; (b) range = 100–1000 ppb; (c) range = 1–5 ppm.

The development of the sample treatments aimed at maximizing the possible injection volume while maintaining an excellent chromatography. This objective was accomplished by maximizing the sample amount and minimizing the amount of acid for sample preparations.

Table 2. Ion Chromatography Analyses of Certified Microwave-Digested and Leached Reference Samples[a]

	Cu	Ni	Zn	Mn	Fe
			Microwave Digestion		
HDP, $n = 12$					
certified values	3.18 ± 0.19	0.271 ± 0.038	28.9 ± 1.3	12.9 ± 0.58	30.4 ± 0.90
a	2.94 ± 0.27	0.311 ± 0.091	28.2 ± 1.9	13.3 ± 2.6	23.4 ± 2.4
c	3.21 ± 0.17	0.300 ± 0.045	28.8 ± 0.71	13.0 ± 0.60	29.7 ± 0.86
diet A, $n = 12$					
certified values	2.60 ± 0.15	0.126 ± 0.056	95.0 ± 4.5	5.69 ± 0.47	81.2 ± 4.8
a	2.27 ± 0.54		95.7 ± 3.4		77.9 ± 5.9
c	2.51 ± 0.11	0.126 ± 0.048	92.2 ± 2.3	5.49 ± 0.28	84.7 ± 5.6
diet E, $n = 12$					
certified values	46.5 ± 1.4	0.124 ± 0.025	39.5 ± 3.1	11.0 ± 0.7	216 ± 17
a	46.4 ± 2.0		38.1± 1.5		218 ± 5
c	49.1 ± 0.90	0.109 ± 0.029	37.8 ± 0.57	11.0 ± 0.6	219 ± 6
animal blood, $n = 8$					
certified values	4.3 ± 0.6		13 ± 1		2400 ± 200
a	3.7 ± 0.5		12.6 ± 0.6		2521 ± 40
b	4.1 ± 0.3		13 ± 0.6		2424 ± 81
c	4.3 ± 0.5		12.8 ± 0.32		nd[b]
			Leaching		
HDP, $n = 4$					
certified values	3.18 ± 0.19	0.271 ± 0.038	28.9 ± 1.3	12.9 ± 0.58	30.4 ± 0.90
b	3.28 ± 1.06	0.438 ± 0.139	29.3 ± 0.10	12.7 ± 0.85	26.9 ± 0.82
c	2.96 ± 0.89	0.256 ± 0.013	25.7 ± 0.56	12.4 ± 0.25	24.5 ± 0.52
diet A, $n = 4$					
certified values	2.60 ± 0.15	0.126 ± 0.056	95.0 ± 4.5	5.69 ± 0.47	81.2 ± 4.8
b	1.89 ± 0.29	0.271 ± 0.097	94.9 ± 0.79	5.20 ± 0.76	81.4 ± 1.3
c	1.73 ± 0.11	0.170 ± 0.064	85.6 ± 1.8	6.06 ± 0.26	75.4 ± 0.49
diet E, $n = 4$					
certified values	46.5 ± 1.4	0.124 ± 0.025	39.5 ± 3.1	11.0 ± 0.7	216 ± 17
b	42.6 ± 0.99	0.190 ± 0.089	39.0 ± 0.45	10.3 ± 0.7	227 ± 1
c	40.7 ± 0.61	0.117 ± 0.022	36.5 ± 0.29	11.3 ± 0.4	213 ± 2

[a] Mean ± SD of replicate samples. Certified values as reported by manufacturers of the reference samples (ppm in dry sample). Sample preparation: 0.4 g of dry weight diluted to 10 mL, (a) 25 μL injection, (b) 50 μL injection, and (c) acid evaporation and dilution of sample to 2.5 mL and then 25 μL injection. [b] nd, not determined.

For samples high in mineral content, a simple water dilution can be applied prior to injection to reduce the effects of acid concentration. Standard solutions originally of 1000 ppm concentration were prepared by diluting the standards to 1000−5 ppb in the solution of 0.05 M HCl and 2 g of ascorbic acid/L.

RESULTS AND DISCUSSION

Detection Limits. Figure 1 shows the chromatogram when 25 μL of a standard solution containing 10 ppb of each mineral was injected. The average detection limit for the minerals corresponds to 5 ppb with a 50 μL injection. At 500 nm wavelength the detection limit is, however, higher for Mn. For the UV−vis absorption a 500 nm wavelength was used because it gave better results compared to the higher wavelengths used in previous publications. The detection limit can perhaps, by using a better pump pulse damper, be further lowered. These detection limits are in agreement with previous studies (*13*, *15*−*17*). The results are better than what normally is obtained with AAS but not as low as the parts per thousand range that is possible by ICP-MS. By using a larger injection volume, up to 1 mL, the detection limit can also be lowered. However, a large injection volume is not possible for acidified samples. Concentrating the sample, as described by Dionex (*3*) and also shown in this paper, can also improve the analysis. A crucial prerequisite to achieve the reported detection limits and to obtain reliable results is to completely exclude all contamination from the sample treatments.

Reproducibility. Series of repeated injections of 50 μL of 10, 50, and 800 ppb standard solutions in mixed order, as well as MW-digested samples of biological origin, were performed to evaluate the stability of the method. The results are shown in Table 1 and indicate satisfactory stability when alternating between the detection limit range and higher concentrations.

Linearity. According to Figure 2 the linearity was excellent for the six minerals. The responses for Cu and Ni are almost identical. The range in Figure 2 covers what one would expect to inject.

Analyses of Certified Reference Samples. The certified reference samples analyzed with IC are reported in Table 2. With HDP, diet A, and diet E we have tried to cover an expected area in food samples. An animal whole blood with a large difference in amounts for different metals was analyzed. In the certified blood sample, which contains a very high amount of iron, there were difficulties in measuring manganese. Coelution is the reason. Possible applications of this method are shown in Table 2 and Figure 3. Depending on which mineral is of interest, one can either concentrate or dilute the sample. The certified reference samples with a great difference in mineral content were used to evaluate the analytical method. Despite this variance in mineral content the results for the reference samples were in agreement with the certified values.

Influence of Sample Treatment. Because of the complexing ability of the PDCA the analytes must be in a free available form, only weakly complexed, or hydrated to be properly chromatographed (*2*). The sample treatments must therefore liberate the metals from their matrices to facilitate the PDCA complexing. Unexpectedly, when Met Pac PDCA eluent and PAR postcolumn reagent from Dionex were used, a much lower response for nickel was observed.

The acidification treatment is a fast and simple preparation, but only for hydrated and weakly complexed soluble minerals

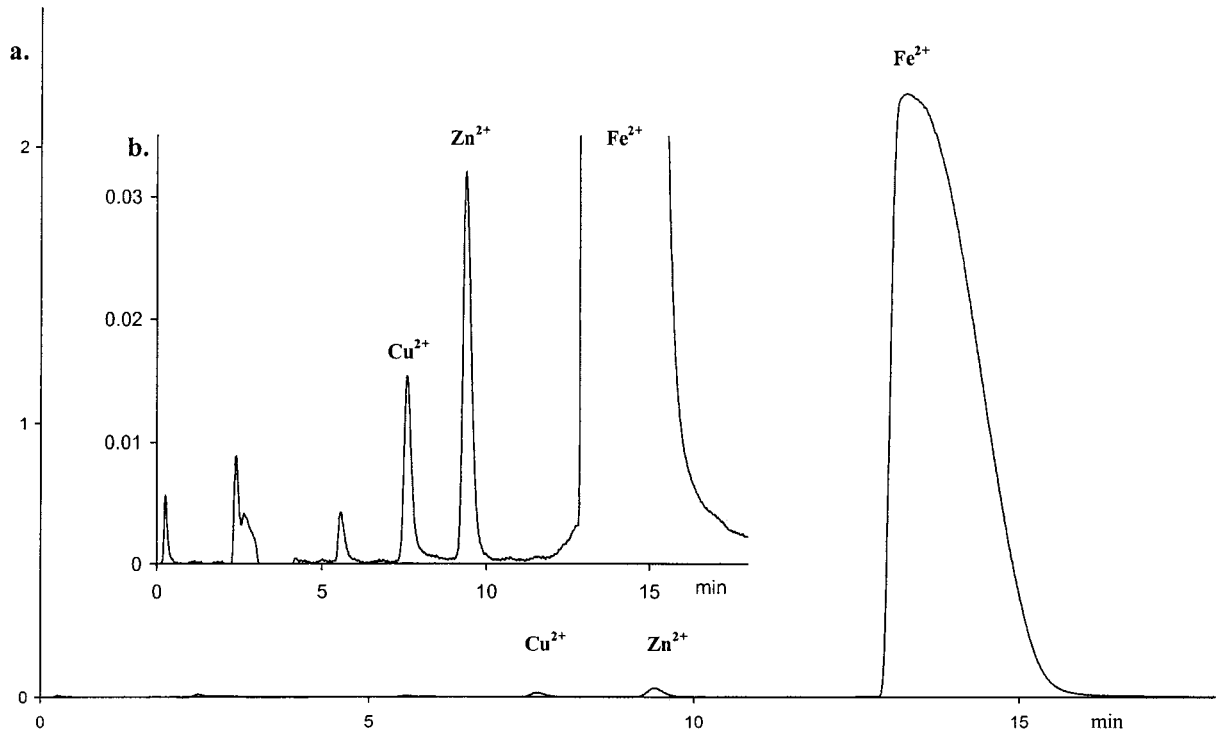

Figure 3. IC analysis of certified animal whole blood: (a) chromatogram normalized for Fe; (b) chromatogram enlarged ~100 times. Injection volume = 25 μL. Microwave digestion of 0.4 g of blood dry weight, evaporated and diluted in 2.5 mL (2 mg/mL ascorbic acid in 0.05 M HCl).

Table 3. Comparison Sample Treatments Using Microwave Digestion and Leaching[a]

	Cu		Zn		Fe	
	MW	leaching	MW	leaching	MW	leaching
animal blood						
a	3.7 ± 0.5	0.90 ± 0.36	12.6 ± 0.6	12.5 ± 0.2	2521 ± 40	1168 ± 7
c	4.3 ± 0.5	0.87 ± 0.13	12.8 ± 0.3	10.4 ± 0.2	1439 ± 44	929 ± 27
	$n = 8$	$n = 4$	$n = 8$	$n = 4$	$n = 8$	$n = 4$
Dona whole maize flour						
a	nd[b]	nd	21.5 ± 0.3	24.7 ± 2.0	31.5 ± 0.4	31.6 ± 1.1
			$n = 3$	$n = 3$	$n = 3$	$n = 3$
sorghum white flour						
a	nd	nd	20.5 ± 1.2	24.6 ± 0.6	170 ± 13	79 ± 4
			$n = 7$	$n = 7$	$n = 7$	$n = 7$
sorghum red flour						
a	nd	nd	nd	nd	48.7 ± 2.2	52.0 ± 1.7
					$n = 6$	$n = 6$
Novo CT granulate						
a	nd	nd	nd	nd	306 ± 32	39.9 ± 0.2
					$n = 6$	$n = 6$

[a] Total copper, zinc, and iron analyses (expressed as ppm in dry sample). Mean ± SD of replicate samples. Sample preparation: 0.4 g of dry weight diluted to 10 mL, (a) 25 μL injection, (c) 25 μL injection, evaporated, and diluted sample to 2.5 mL. [b] nd, not determined.

in liquid samples. The concentration by evaporated volume reduction was suitable for liquid samples low in the analytes (Table 2).

A comparison of MW digestion and leaching is shown in Table 3. For certain samples, different results were obtained depending on whether the leaching or the MW digestion was used. The sample matrix and the composition of the mineral-containing compounds greatly influence both the rate and extent of mineral extraction. The leaching procedure is shown to be inadequate for extracting the total content of minerals in samples in wihch the metals are bound as insoluble inorganic matrices, as observed with Novo CT granulate enzyme and white sorghum flour. The Novo CT granulates phytase enzyme consists of the enzyme with sodium sulfate and crystalline cellulose as carrier materials. The granulate is triple-coated with kaolin (porcelain clay) and hydrogenated palm oil. The mineral contamination in the granulates detected by MW digestion is located in the kaolin, which mainly consists of $Al_2(OH)_4Si_2O_5$. The different results obtained for white sorghum flour are explained by the open-air sun-drying procedure, which allows inorganic mineral contamination to enter the flour from the soil and by the wind. For the other samples including the reference samples the leaching treatment gave acceptable results. Thus, the leaching procedure is recommended for analyzing biologically bound weakly complexed minerals.

Too high acid content in the injected sample from the sample treatment causes a negative peak that negatively influences the chromatography. This effect is especially noticed when nitric acid (HNO_3) is used. Due to this artifact it is important to ensure that the acid (H^+) concentration in the injected sample is as low as possible, ~0.05 M. The chromatography of manganese and sometimes iron is especially sensitive to the acid concentra-

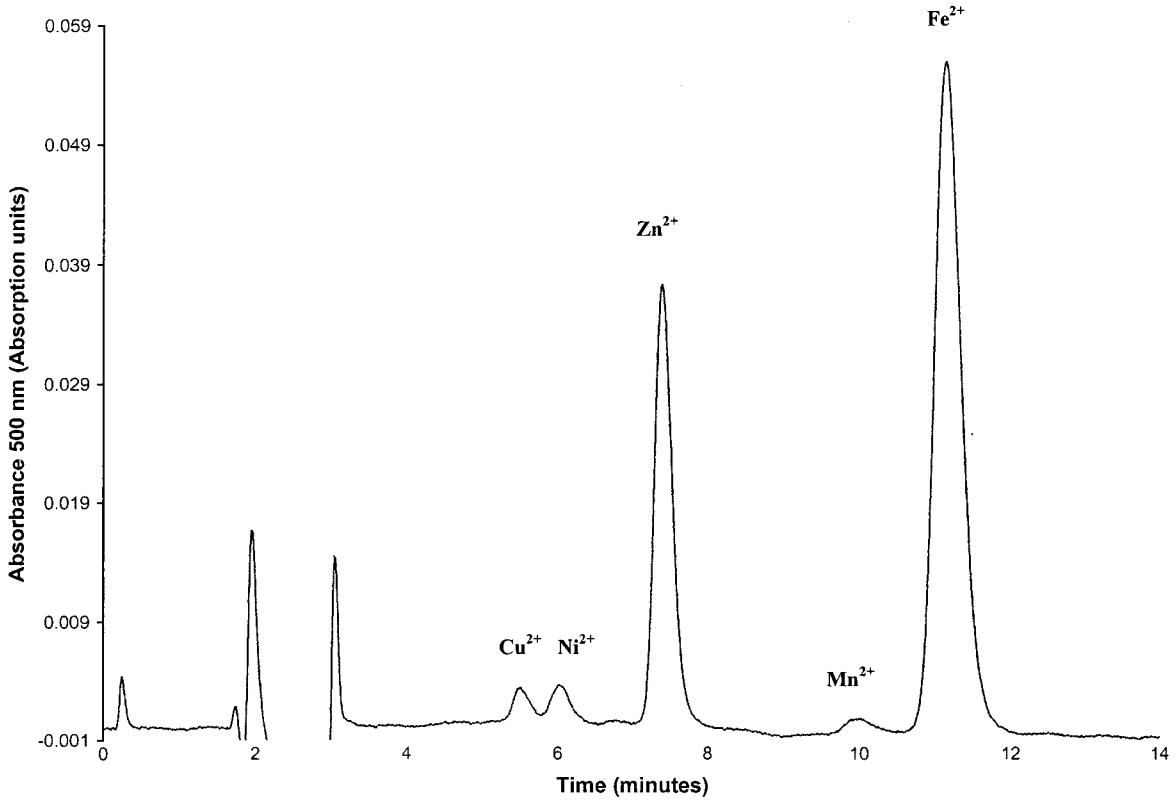

Figure 4. Typical chromatogram of a microwave-digested sample of food origin. Sample preparation consisted of 0.4 g of dry weight diluted to 10 mL and a 25 μL injection.

tion. To avoid this nitric acidic propensity in samples with low concentrations of minerals, evaporation and redissolving with 0.05 M HCl are used. This is less important when samples with a high mineral content are digested, because with a high mineral content in the samples the acidic effect can be reduced by a dilution before injection. Use of an acidified standard solution to analyze acid samples is recommended. Figure 4 shows a chromatogram from the injection of a MW-digested acidic sample (25 μL injected) of food origin.

The sample treatment can be chosen according to the sample type and whether the quantification of total mineral content, biologically bound mineral content, or soluble mineral content is wanted.

pH Influence for the PAR Complexation of Metals. The postcolumn complexation of the metals by PAR is dependent on the pH value. As the pH increases, the PAR dissociation increases, which is necessary for the complexation of the metals. However, at higher pH the metal ions hydrolyze, which inhibits the complexation with PAR. By adjustment of the pH the metal−PAR complexation can be customized, and by this additional metal species, for example, Pb, Cd, and Hg, can be detected by PAR complexation (*8*, *23*).

Conclusion. Compared to AAS and ICP the method presented here is fast and sensitive and allows simultaneous determinations of six minerals in food and other biological samples, requiring small sample volumes. Thus, it is a preferable and also less expensive alternative to AAS and ICP.

ABBREVIATIONS USED

AAS, atomic absorption spectroscopy; IC, ion chromatography; ICP, inductively coupled plasma; ICP-MS, inductively coupled plasma−mass spectroscopy; i.d., inner diameter; MW, microwave; MWCO, molecular weight cutoff; PAR, 4-(2-pyridylazo)resorcinol; PEEK, polyether ether ketone; PDCA, pyridine-2,6-dicarboxylic acid.

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Received for review June 21, 2001. Revised manuscript received October 22, 2001. Accepted October 22, 2001. This work was supported by the Swedish Nutrition Foundation.

JF010792W