



# Zinc determination in human milk by flameless atomic absorption spectrometry after dry ashing

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A zinc determination method, using a dry ashing step and flameless atomic absorption spectrometry is described. Milk samples were dry ashed at 400°C. Then, the ash was dissolved in 0.1 M nitric acid (final dilution = 1 + 99). The within-run precision was found to be 8%. The between-run was found to be 5%. The recovery of standard addition was found to be 109 ± 9%. The linear range was 122 nmol litre<sup>-1</sup> to 2 µmol litre<sup>-1</sup>. The analytical variables (dry ashing temperature, dilution rate, acid, furnace, calibration) are discussed. A significant loss of zinc was observed at temperature <450°C. The dilution rate and acid used for dissolution of dry ash had an important effect on the validity of the method. A comparison of the proposed method with a direct method without an ashing step was performed. The results obtained from these two methods were similar. From these results, it is concluded that flameless atomic absorption spectrometry after dry ashing is a reliable technique for the determination of zinc in solid foods or tissues.

## INTRODUCTION

Human milk is the best source of nutrients for infants. However, human milk zinc concentration decreases rapidly with progressive lactation. Moreover, a few cases of zinc deficiency in premature or full-term infants fed with their own mothers' milk have been reported (Bye *et al.*, 1985; Murphy *et al.*, 1985; Kuramoto *et al.*, 1986; Roberts *et al.*, 1987; Zeigler, 1985). In these cases, milk zinc concentration was particularly low (1.4–7.6 µmol litre<sup>-1</sup>). As a result, a sensitive method for zinc determination in milk is necessary.

Methods used for zinc determination in human milk include colorimetry (Chauhan *et al.*, 1981), fluorometry (Moreno *et al.*, 1983), polarography (Sann *et al.*, 1981), emission spectroscopy (Feeley *et al.*, 1983; Schramel *et al.*, 1988), X-ray fluorescence spectrometry (Palma *et al.*, 1983), neutron activation (Dang *et al.*, 1982; Kosta

*et al.*, 1983; Lavi & Alfassi, 1990), but the most commonly used method is flame atomic absorption spectrometry. Some of these methods have a poor sensitivity. To the best of our knowledge, flameless atomic absorption spectrometry, which is very sensitive, has only been used by Saner and Yuzbasiyan (1984) for zinc determination in milk.

Zinc determination in biological media or foods sometimes needs a previous step of matrix simplification or dissolution. Wet and dry ashing procedures are the most commonly used. The two main disadvantages of the former are incomplete digestion if there are high concentrations of lipids in the sample and the use of large volumes of acids, which may lead to interferences and reduced graphite furnace tube life (Lonnerdal *et al.*, 1980; Taylor, 1988). Casey *et al.* (1989) have used a low-temperature asher, which is probably the best technique. The only drawback to this method is the cost of the low-temperature asher. Dry ashing muffle furnaces are used in several reports. A temperature of 360°C has been used by Costantini *et al.* (1981), whereas Picciano (1978), Rajalaskmi and Srikantia (1980) and Lippolis *et al.* (1989) have used a

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temperature of 550°C. However, contamination (Byrne *et al.*, 1979; Clegg *et al.*, 1981) or losses of metal (Sandell & Onishi, 1978; Byrne *et al.*, 1979; Rajalaskmi & Srikantia, 1980; Taylor, 1988) have been described. Losses of zinc have been explained by a zinc volatilization or by an adsorption of metal on crucibles (Sandell & Onishi, 1978; Byrne *et al.*, 1979; Clegg *et al.*, 1981; Taylor, 1988). Quartz has been used by Schramel *et al.* (1980) and Sandell and Onishi (1978) to overcome the adsorption phenomenon. Nevertheless, the effect of temperature on zinc losses has not been comprehensively examined.

Dry ash is dissolved in hydrochloric (Murthy, 1974; Picciano, 1978; Byrne *et al.*, 1979; Vaughan *et al.*, 1979; Arpadjan & Nakova, 1981; Saner & Yuzbasiyan, 1984; Casey *et al.*, 1989) as well as in nitric acid (Murthy, 1974; Costantini *et al.*, 1981; Moran *et al.*, 1983). To the best of knowledge, a comparison of these two acids for zinc determination in milk has not been previously reported.

This work describes a sensitive method for zinc determination in human milk, by flameless atomic absorption spectrometry, after a dry ashing step. Loss of zinc during dry ashing is studied. Performances of hydrochloric and nitric acid for ash dissolution are compared, in terms of accuracy and precision. A comparison of non-coated and pyrolytically coated graphite furnace tubes is also made. Finally, zinc concentrations in human milk obtained from the proposed method are compared to milk zinc concentrations obtained from a direct method.

## MATERIALS AND METHODS

### Apparatus

A Perkin Elmer model 560 fitted with a HGA 500 graphite furnace and a AS 40 autosampler was used. The energy of the hollow cathode lamp was adjusted to 15 mV. The wavelength absorption line and slit width used were 213.9 nm and 0.7 nm, respectively. The temperature, ramp time and hold time of the three steps of the graphite furnace programme were: Dry: 110°C/20 s/20 s, ash: 650°C/1 s/30 s, atomization: 2200°C/1 s/5 s. The nitrogen flow during the atomization step was reduced to 250 ml min<sup>-1</sup>. Standard (non-coated) graphite and pyrolytically coated graphite furnace tubes were used. The injection volume in the graphite furnace was 10 µl.

### Materials

All glassware, polystyrene tubes and quartz crucibles were soaked in 10% (v/v) nitric acid overnight in order to eliminate zinc contamination. Then, they were immersed in 10% (v/v) hydrochloric acid until required,

to avoid any further contamination. Finally, they were rinsed several times with deionized water before analysis.

### Reagents

Hydrochloric acid (0.1 M and 1 M) was prepared with ultrapure concentrated hydrochloric acid (Normaton, Prolabo, Paris, France). Nitric acid (0.1 M and 1 M) was prepared with ultrapure nitric acid (Merck, Darmstadt, West Germany). 0.1% (w/v) Triton X 100 is another reagent (Prolabo, Paris, France).

The zinc stock standard solution (75 µmol litre<sup>-1</sup>) was prepared by means of zinc metal (Merck, Darmstadt, West Germany). First, 4.9 mg of zinc metal were dissolved in 5 ml of concentrated hydrochloric acid. Then, this solution was poured in a 1 litre volumetric flask containing about 700 ml of deionized water. Finally, the volume was adjusted with deionized water. The working standard zinc solutions were prepared by dilution in the acid solution used for ash dissolution. The concentration of these standard solutions, expressed in µmol litre<sup>-1</sup> of milk were 37.5, 75 and 150.

### Samples

The breast was cleaned with deionized water. Milk was collected by manual expression of the breast, before one of the morning feeds (9 to 11 a.m.). Approximately 10 ml of milk were collected directly into a 30 ml polystyrene tube. One millilitre aliquots were immediately frozen at -20°C in 5 ml polystyrene tubes. Samples were stored frozen until analysis.

### Assay

Two hundred microlitres of milk and 200 µl of deionized water or one of the working standard solutions were placed in a quartz crucible. Crucibles were placed in a muffle furnace. After drying at 105°C for about 1 h, the muffle furnace temperature was increased to 400°C. This temperature was maintained for about 16 h, in order to obtain white ash. The ash was dissolved in 20 ml of 0.1 M nitric acid. Ten microlitres of this solution were injected in the graphite furnace.

### Calculations and statistical analysis

The detection limit was calculated according to the following formula:  $m + 3 SD$ , where  $m$  was the mean of 30 replicate zinc determinations at the blank level (0.1 M nitric acid) and SD was the corresponding standard deviation. The linear upper limit was defined as the inflexion point of the calibration curve (three measurements). The within-run precision was expressed as the coefficient of variation of 10 different determinations of the same milk sample, performed the same

day. The between-run precision was the coefficient of variation of 20 zinc determinations of the same milk sample, performed over a one month period. The accuracy was defined as the mean percentage of recovery of three standard additions per sample, obtained from 20 zinc determinations of the same milk, performed on 20 different days.

Zinc was determined in 30 milk samples using the proposed method and a direct method previously described (Arnaud, 1987). This direct method used a simple dilution of milk samples in 0.1% Triton  $\times$  100 before injection in a graphite furnace tube. The Student *t*-test, linear regression and correlation coefficient were used to compare both methods.

## RESULTS AND DISCUSSION

### Effect of ashing temperature

The effect of ashing temperature was evaluated on the precision, accuracy, losses of zinc and duration of analysis. For this test, the ash was dissolved in 40 ml 0.1M hydrochloric acid, which is the most commonly used (Murthy, 1974; Picciano, 1978; Byrne *et al.*, 1979; Vaughan *et al.*, 1979; Arpadjan & Nakova, 1981; Saner & Yuzbasiyan, 1984; Casey *et al.*, 1989). Zinc losses were detected by differences between zinc concentrations obtained from two external calibration curves. The first one was treated according to sample preparation, whereas the second one was prepared by dilution of the zinc standard solutions in 0.1M hydrochloric acid. The results are presented in Table 1 and Fig. 1.

Zinc losses became significant at 450°C (Fig. 1). This result was unexpected, because of the use of a temperature  $\geq$ 450°C in many reports (Murthy *et al.*, 1972; Casey, 1977; Picciano, 1978; Sandell & Onishi, 1978; Byrne *et al.*, 1979; Vaughan *et al.*, 1979; Vuori, 1979; Rajalaskmi & Srikantia, 1980; Mendelson *et al.*, 1983;

Moran *et al.*, 1983). Moreover, Van Raaphorst *et al.* (1974) have described significant zinc losses only at an ashing temperature up to 1000°C. However, Rajalaskmi and Srikantia (1980) have observed significant zinc losses at 550°C.

The ashing temperature had no effect on precision (Table 1). The coefficients of variation were similar to those obtained by flame atomic absorption spectrometry (Murthy *et al.*, 1972; Vuori, 1979).

The recoveries of standard additions, calculated from the first calibration curve, were <90% (Table 1). Recoveries obtained by Mendelson *et al.* (1983), Moran *et al.* (1983), Murthy *et al.* (1972) and Saner and Yuzbasiyan (1984), after dry ashing at 450°C, varied from 96.4 to 98%; whereas those reported by Rajalaskmi and Srikantia (1980), after dry ashing at 550°C, were less than 10%. It was not possible to explain the lower recoveries observed after ashing at 350°C and 550°C.

Zinc concentrations, calculated from the first calibration curve (Table 1), decreased slightly, while temperature increased. This decrease became significant at 550°C ( $p < 0.001$ ).

In conclusion, 400°C was chosen as a result of no significant zinc losses and only an overnight ashing step. However, the standards must be treated in the same way as the samples, to avoid any erroneous results. The experiments designed to optimize the other parameters (e.g. diluent, dilution rate etc.) were conducted at this temperature.

### Effect of dilution factor

The dilutions were selected according to the zinc concentration in milk and the signal to noise ratios (Table 2).

Table 1. Effect of the dry ashing temperature on the accuracy, precision and ashing time

Temperature (°C)	Zinc ( $\mu\text{mol litre}^{-1}$ )	Standard addition recovery (%)	Between-run precision (%)	Ashing time (h)
350	105 $\pm$ 6	59.5 $\pm$ 7	5.7	32
400	103 $\pm$ 6	81.5 $\pm$ 7	5.8	16
450	100 $\pm$ 4.5	83.5 $\pm$ 5	4.5	4
500	104 $\pm$ 3	78 $\pm$ 6	2.9	2
550	89 $\pm$ 3*	66.5 $\pm$ 10	3.4	1
600	96 $\pm$ 5	88.5 $\pm$ 6	5.2	1

The ash was dissolved in 0.1M hydrochloric acid. The final dilution was 1 + 199. The results are expressed as mean  $\pm$  1 SD ( $n = 20$  zinc determinations in the same milk but in different series).

\* $p < 0.001$ .

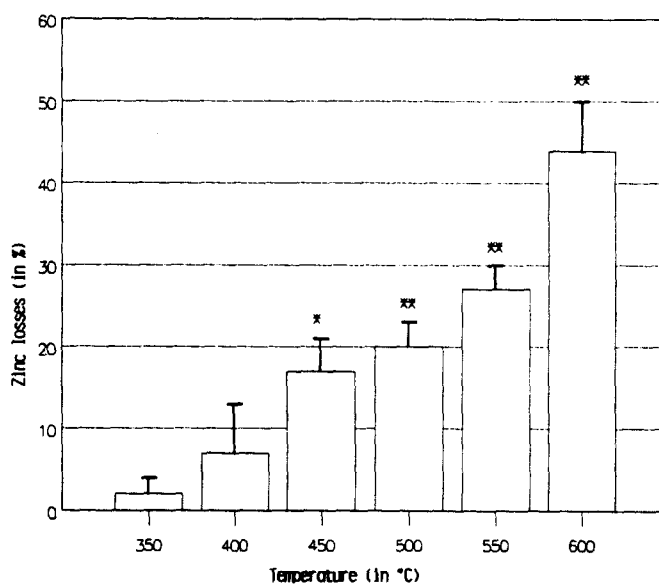


Fig. 1. Influence of the ashing temperature on the zinc losses. The results are expressed as mean  $\pm$  1 SD ( $n = 20$ ). \*\* $p < 0.001$ , \* $p < 0.01$ .

**Table 2. Effect of the acid and dilution rate on the precision and accuracy**

	Dilution rate		
	1 + 49	1 + 99	1 + 199
Accuracy (recovery of standard additions in %)			
HCl	43.5 ± 4 <sup>b</sup>	72 ± 5 <sup>b</sup>	81.5 ± 7 <sup>b</sup>
HNO <sub>3</sub>	89 ± 6 <sup>a,b</sup>	104.5 ± 5 <sup>b,b</sup>	132.5 ± 5 <sup>b</sup>
Between-run precision (%)			
HCl	6.4	4.7	5.8
HNO <sub>3</sub>	3.7	4.7	4.5
Zinc concentration (μmol litre <sup>-1</sup> )			
HCl	78 ± 5 <sup>b,b</sup>	110 ± 5 <sup>b</sup>	103.5 ± 6
HNO <sub>3</sub>	108 ± 4 <sup>b</sup>	105.5 ± 5	110 ± 5
Signal to noise ratio			
HCl	28 ± 6	15 ± 3	8 ± 4
HNO <sub>3</sub>	34 ± 6	17.5 ± 4	7 ± 1

The results are expressed as mean ± 1 standard (20 zinc determinations in the same milk but in different series).

<sup>a</sup>*p* < 0.01.

<sup>b</sup>*p* < 0.001.

The final dilution had a great effect on the recovery of standard additions, probably related to the linear range (Table 2). However, the dilution factor had only a slight effect on the precision (Table 2). Likewise, the dilution factor had generally no effect on zinc concentrations, calculated from an external calibration curve (Table 2). However, with a final dilution (1 + 49) in 0.1M hydrochloric acid, zinc concentrations were significantly reduced (*p* < 0.001), probably because of the significantly reduced recovery of standard additions. In a collaborative study, similar results have been obtained for zinc determination in serum, by flame atomic absorption spectrometry (Arnaud *et al.*, 1986).

#### Effect of acid nature

The dry ash was dissolved in hydrochloric acid 0.1M, as well as in nitric acid 0.1M. Zinc contamination of nitric acid was higher than that of hydrochloric acid (HNO<sub>3</sub>: 77 ± 15 nmol litre<sup>-1</sup>; HCl: 46 ± 15 nmol litre<sup>-1</sup>; *p* < 0.001).

The use of nitric acid slightly reduced zinc loss (HNO<sub>3</sub>: 4 ± 1%, *n* = 10; HCl: 7 ± 6%, *n* = 20).

Nitric acid (0.1M) allowed a better recovery of standard additions (Table 2). It was not possible to present any explanation for this phenomenon. However, for zinc chloride and zinc nitrate, the atomization mechanism is different (Pinta, 1979). Moreover, chloride and nitrate matrix effect may explain these results. The influence of the acid used is particularly well known for cadmium and lead determination (Murthy, 1974;

Taylor, 1988). Nevertheless, for zinc determination in milk, hydrochloric acid is still used.

With a dilution as large as 1 + 99 or 1 + 199, 0.1M nitric acid or hydrochloric acid had no effect on zinc concentrations, calculated from external calibration curve (Table 2); whereas, with 1 + 49 dilution, zinc concentrations were significantly (*p* < 0.001) lower with 0.1M hydrochloric acid. These lower concentrations might be explained by the poor recovery of standard additions with 0.1M hydrochloric acid.

On the contrary, the nature of the acids had no influence on the precision (Table 2). A dilution of 1 + 99 in 0.1M nitric acid allowed the best accuracy and an acceptable precision. In agreement with this choice, Constantini *et al.* (1981), Mendelson *et al.* (1983) and Moran *et al.* (1983) dissolved the ash in nitric acid for zinc determination in milk.

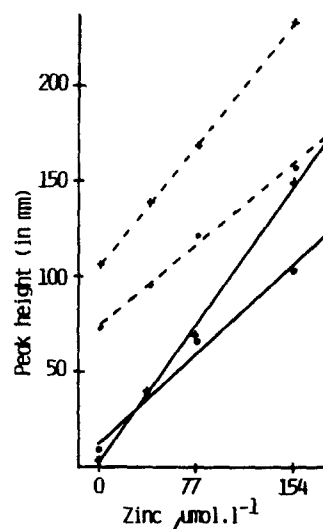
#### Influence of graphite furnace tubes

Non-coated and pyrolytically coated graphite furnace tubes were evaluated.

The responses for aqueous standards and standards added to diluted milk ash were enhanced when using standard tubes (Fig. 2). These results are different from those of Foote and Delves (1982) and Sturgeon and Chakrabarti (1977).

The detection limit was better when using uncoated graphite furnace tubes (122 nmol litre<sup>-1</sup>) than when pyrolytically coated graphite furnace tubes were used (140 nmol litre<sup>-1</sup>). These results are different from those of Sturgeon and Chakrabarti (1977).

The accuracy was similar. The average recoveries of



**Fig. 2.** Effect of the graphite furnace tube on the zinc signal. The results are expressed as mean (*n* = 10 determinations of the same milk sample on different days). +—+, Uncoated graphite furnace tube (aqueous standards); ●—●, pyrolytically coated graphite furnace tube (aqueous standards); +----+, uncoated graphite furnace tube (milk sample); ●----●, pyrolytically coated graphite furnace tube (milk sample).

standard additions, obtained from 10 determinations of the same milk sample on different days were  $109 \pm 8\%$  with standard non-coated graphite furnace tubes and  $100 \pm 62\%$  with pyrolytically coated graphite furnace tubes.

The corresponding zinc concentrations were not significantly different. Zinc concentrations, expressed as mean  $\pm$  one SD were found to be  $111 \pm 5 \mu\text{mol litre}^{-1}$  with standard non-coated graphite furnace tubes, whereas, with pyrolytically coated graphite furnace tubes, they were found to be  $100 \pm 5 \mu\text{mol litre}^{-1}$ .

On the contrary, the within-run precision, obtained from 10 determinations of the same milk sample, performed in the same conditions, was better with a standard non-coated graphite furnace tube. The coefficient of variation was 8% with a standard graphite furnace tube and 12% with a pyrolytically coated graphite furnace tube. These results were in agreement with those of Sturgeon and Chakrabarti (1977).

Consequently, it is recommended, as it was by Foote and Delves (1982), for zinc determination in serum, that standard graphite furnace tubes are used for zinc determination in milk.

#### Influence of calibration process

Zinc in 40 human milk samples was measured using two calibration procedures: an external calibration curve and the standard additions method. The results are presented in Fig. 3. Mean zinc concentrations were  $108 \mu\text{mol litre}^{-1}$  (range:  $31\text{--}357 \mu\text{mol litre}^{-1}$ , SD:  $66 \mu\text{mol litre}^{-1}$ ) using an external calibration and  $115 \mu\text{mol litre}^{-1}$  (range:  $26\text{--}274 \mu\text{mol litre}^{-1}$ , SD:  $65 \mu\text{mol litre}^{-1}$ ) using the standard additions method. Although, differences were not statistically significant, the correlation coefficient was poor ( $r = 0.71$ ). The linear regression was:

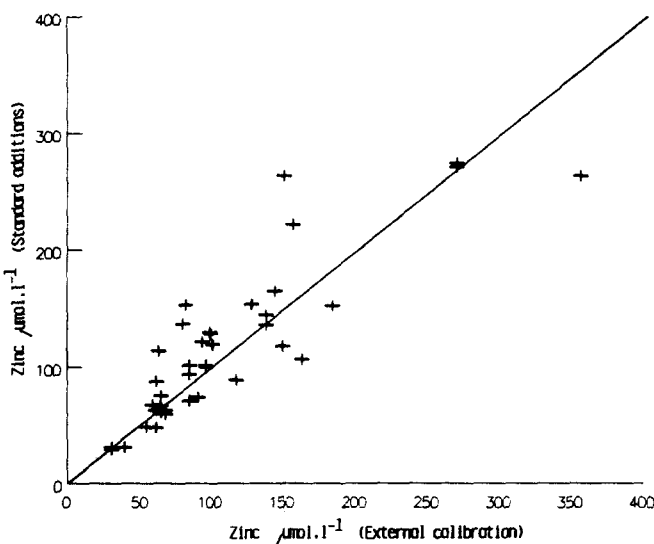


Fig. 3. Influence of the calibration process on milk zinc concentration. The solid line was the regression line through the results (intercept =  $0 \mu\text{mol litre}^{-1}$ ).

$$\text{Zn}_{(\text{Standard additions})} = 1.00_{(\text{External calibration})} + 0 \mu\text{mol litre}^{-1}$$

The determination of zinc in milk samples may give different results when using the two calibration methods, depending on the matrix of milk. Considerable variability in mineral concentrations between women have been described (Atkinson *et al.*, 1980). The standard addition slopes (peak height in mm/concentration in  $\mu\text{g litre}^{-1}$ ) varied from 1.18 to 3.44 (mean  $\pm$  1 SD:  $1.72 \pm 0.23$ ,  $n = 40$ ), whereas aqueous standard slope varied from 1.31 to 2.04 (mean  $\pm$  1 SD:  $1.68 \pm 0.11$ ,  $n = 30$ ). The recoveries of standard additions were found to be  $102 \pm 37\%$  (range: 49–168%,  $n = 40$ ). As a result, the standard additions method was necessary. Moran *et al.* (1983), using flame atomic absorption spectrometry, recommend the use of standard additions to determine zinc concentrations less than  $13 \mu\text{mol litre}^{-1}$ .

#### Comparison of the proposed method with a direct method

Zinc concentrations were calculated in 30 milk samples, by the standard additions method. The linear regression is presented in Fig. 4. The broken line is the line of identity and the solid line is the regression line through the results. The regression was:

$$\text{Zn}_{(\text{Proposed method})} = 0.90_{(\text{Direct method})} + 0 \mu\text{mol litre}^{-1}$$

with an associated correlation coefficient of 0.95. The mean zinc concentration by the proposed method was found to be  $136 \mu\text{mol litre}^{-1}$  (range:  $27\text{--}274 \mu\text{mol litre}^{-1}$ , standard deviation  $61 \mu\text{mol litre}^{-1}$ ). The mean zinc concentration by the direct method was found to be  $147 \mu\text{mol litre}^{-1}$  (range:  $46\text{--}315 \mu\text{mol litre}^{-1}$ , standard deviation:  $73 \mu\text{mol litre}^{-1}$ ). Differences between the two methods were not statistically significant. These results

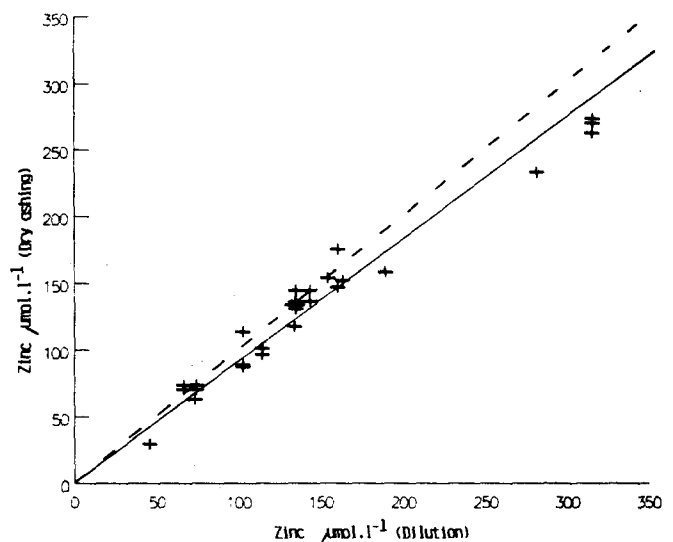


Fig. 4. Comparison of the proposed method with a direct flameless atomic absorption method. The broken line was the line of identity. The solid line was the regression line through the results (intercept =  $0 \mu\text{mol litre}^{-1}$ ).

are in agreement with previous studies, using flame atomic absorption spectrometry (Rajalaskmi & Srikantia, 1980; Arpadjan & Nakova, 1981; Moran *et al.*, 1983).

#### Validity of the proposed method

The detection limit was found to be 122 nmol litre<sup>-1</sup>, whereas, the calibration curve was linear up to 2 µmol litre<sup>-1</sup>.

The within-run precision was found to be 8%, whereas the between-run precision was found to be 5%.

The accuracy was estimated by means of the standard addition recoveries and by a comparison with a direct method. The recoveries of the standard additions were found to be 109 ± 8% (*n* = 20, pooled milk). Moreover, there were no significant differences for milk zinc concentrations, when using the proposed method and a direct method. However, the standard addition recoveries, obtained from 40 milk samples, varied from 49 to 168%. As a result, standard additions for each separate sample were necessary. The accuracy was also checked against National Institute of Standards and Technology Standard Reference Material 1549 Non-Fat Milk Powder. The results were 702 ± 22 µmol g<sup>-1</sup> (*n* = 10) (certified value: 705 µmol g<sup>-1</sup>).

#### CONCLUSIONS

The validity of the proposed method is adequate for biochemical investigations. Nevertheless, the proposed method is time consuming, allows only few determinations per series and matrix effects make the use of the standard addition procedure necessary. Furthermore, differences in zinc concentrations obtained from the proposed method and a direct method are not statistically significant. The authors think, as Arpadjan and Nakova (1981), Moran *et al.* (1983) and Rajalaskmi and Srikantia (1980), that a direct method is more useful for zinc determination in human milk.

Nevertheless, this study clearly demonstrates that zinc loss occurs, even at temperatures <450°C. Likewise, the importance of the dilution rate and acid used for ash dissolution, on the validity of the method is demonstrated. Finally, uncoated graphite furnace tubes prove to be more suitable than pyrolytically coated graphite furnace tubes, as reported by Foote and Delves (1982) for zinc determination in serum. These observations must be taken into account for determining low zinc concentrations, when a direct analysis is impractical (e.g. solid foods or tissues).

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