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An Enzymatic Method for Determination of Zinc in Milk and Dairy Products

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In this study, an enzymatic zinc determination method based on the regaining of the activity of apo carbonic anhydrase has been presented. The method was applied to some milk and its products to determine the amount of zinc. For this purpose, milk, cheese, yogurt, ice cream and butter in Erzurum were taken and the zinc concentrations were determined with an enzymatic method. Carbonic anhydrase was purified by a Sepharose-4B-L tyrosine sulphanilamide affinity chromatography from bovine erythrocytes. The zinc, in this enzyme, was removed by dialysis against dipicolinic acid resulting in apo carbonic anhydrase was obtained at a ratio of 100 %. The regaining activity of the enzyme was determined by the esterase activity on *p*-nitrophenylacetate. For comparison, the same samples were analyzed in an atomic absorption spectrophotometer (AAS). When the result of the AAS were compared with the enzymatic method, a positive correlation between two methods was observed. This suggests that enzymatic methods can be used in some milk and dairy products.

Key Words: Milk, Cheese, Yogurt, Ice cream, Butter, Zinc determination, Apo carbonic anhydrase.

INTRODUCTION

Some enzymes have had applications in analytical chemistry because of their substrate specificity[1,2]. Metallo-enzymes function by means of the metal ions located in their active sites. The ions are bound to the enzymes specifically, giving rise to an analytical basis for the determination of the metal ion in question. Some studies have been made using amino peptidase, alkaline phosphatase and polyphenol oxidase for the determination of trace elements such as zinc and copper[3,4].

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Carbonic anhydrase (E.C.4, 2,1,1; CA: carbonate hydrolyze) is a zinc, containing metallo-enzyme, which catalyzes hydration of CO_2 and dehydration of H_2CO_3. When the zinc covalently bound to active site is removed, the apocarbonic acid (apoCA) is obtained, resulting in the deactivated enzyme[5]. The apoCA can show activity when the Zn^{2+} is added to the reaction medium, which is proportional to Zn^{2+} added and this is the basic principle of the method. This method was first tried by Kobayashi *et al.*[6] They used the Zn^{2+} in milk and dairy products and water for the reactivation of apoCA and determined the activity by means of esterase action. However, they used 1,10-phenanthroline as chelating agent and could not obtain the apoenzyme at a ratio of 100 %. They could not remove entirely the native Zn^{2+} present on the enzyme. Küfrevioglu and Keha[7] tried a different chelating agent, dipicolinic acid and achieved a more purified enzyme (97 %) in a short time (3 h). They used urine cerebrospinal fluid and serum as samples and inactivated the CA present in serum by boiling it. Recently Demir *et al.*[8] could obtain the enzyme at a high purity (100 %) by longing the dialysis time up to 5 h and they tried the method in different samples obtained from the patients with diabetes mellitus and cirrhosis.

Recently, Demir *et al.*[9-11] obtained the enzyme at a high purity (100 %)[5]. This method was used to determine the zinc level in the Pleural fluid, fruit juice and vegetable.

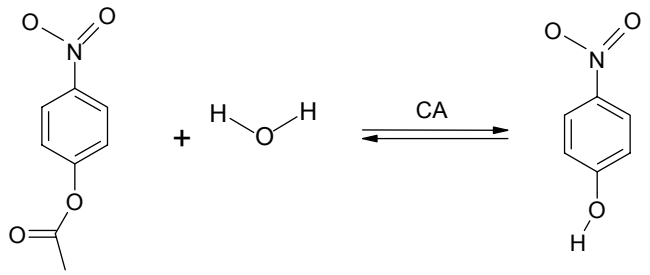
In order to show the application of a new enzymatic method for zinc determination in milk and dairy products, the present study was conducted with a t-test for milk and dairy products material.

EXPERIMENTAL

Preparation of apoCA from bovine erythrocytes: Carbonic anhydrase (CA) was purified by a Sepharose-4B-L tyrosine sulphanilamide affinity chromatography from bovine erythrocytes[12]. For obtaining apo enzymes, carbonic anhydrase was dialyzed against 0.075 M dipicolinic acid. By using this method almost 100 % pure apoenzyme was obtained. 5 mL of the apo enzyme reagent can be prepared by using 100 mg of carbonic anhydrase. The resulting reagent may be sufficient for about 100 determinations. In addition, carbonic anhydrase is a rather stable enzyme and has a long reconstitution life[13]. For instance, only 5 % of activity loss is detected after one year standing.

Carbonic anhydrase activity in eluates obtained during the purification was determined by the method of Wilbur and Anderson as modified by Rickly *et al.*[14,15]. For the preparation of a standard curve in serum, Zn^{2+} determination, the esterase activity of CA was used. In this method, 4-nitrophenyl acetate is hydrolyzed to 4-nitrophenol by CA and the absorbance of the product is measured at 348 nm. Reaction mixtures in a 3 mL

cuvette contained 0.1 mL of apoenzyme solution, 1.0 mL of *tris*-H_2SO_4 (0.05 mol/L, pH: 7.4), 0.4 mL of serum (or standard) and 1.5 mL of substrate. After 3 min, the absorbance's of the sample and blank cell (it contains distilled water instead of sample) were measured at 348 nm at 25 ºC[16].



4-Nitrophenyl acetate 4-Nitrophenol

The *p*-nitrophenyl acetate solution was prepared by dissolving 27.2 mg of ester in 1 mL of acetone and then adding this to 49 mL of distilled was added drop wise to it.

Preparation of milk and dairy products extract: Used samples of milk and dairy products was bought from markets. Two different marks of each product was bought. In each product, this measuring was done three times. The casaine was collapsed by pH and adjusted to the level of 4.8 (isoelectric point) from milk and dairy products which were mixed with physiological salina (NaCl 0.9 %). After it was centrifuged (10000 rpm, 0.5 h, 4 ºC), collapsed casaine was discarded. Then the supernatante was extracted by CCl_4 to eliminate the lipids from the medium and this was followed by the removal of organic media. Finally, in the medium, all of the proteins were collapsed with TCA (8 %) and then centrifuged for 15 min at 10000 rpm. The pH of the supernatante was adjusted to 7.4. This procedure was applied to milk, cheese, ice cream and yogurt. Butter was solved in the CCl_4. It was mixed thoroughly with the same volume water. The water phase was separated and Zn^{2+} was determined in this phase.

Preparation of standard curves: A standard stock solution of Zn^{2+} was prepared by dissolving the zinc metal in sulfuric acid. This solution was diluted and adjusted to a pH of 7.4 by adding *tris*- H_2SO_4 for the preparation of a standard curve. The relationship between apoCA and Zn^{2+} concentrations was determined by the measuring activities at different Zn^{2+} concentrations with a fixed amount of apoCA (3.6×10^{-5} M). A graphical representation of absorbance *vs.* Zn^{2+} concentration was given in Fig. 1.

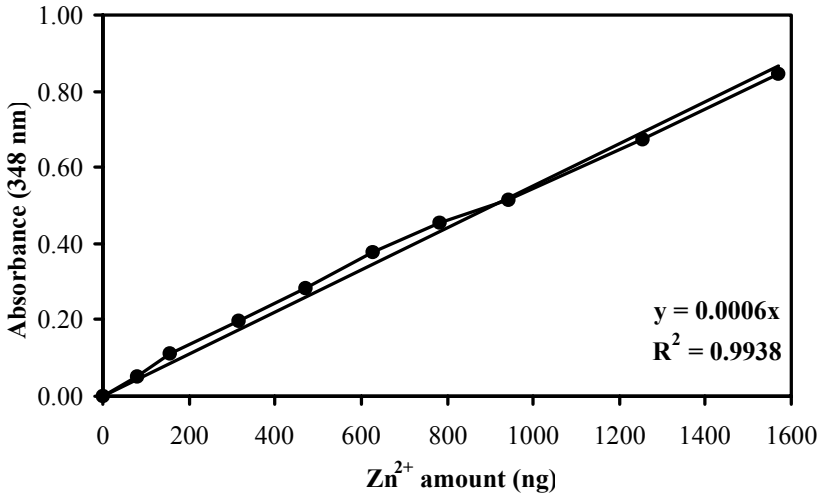


Fig. 1. Standard curve obtained with constant apoCA (3.6×10^{-5} mol/L) and changing Zn^{2+} concentrations

Zinc determination in milk and dairy products: In order to show the applicability of this method to Zn^{2+} determination of milk and dairy products, measurements were made on milk, cheese, yogurt, ice cream, butter samples taken from Erzurum. The water was heated in a boiling water bath for 1 h in test tubes and then was centrifuged. The samples (1 mL) were diluted with deionized water (3 mL) and they were deproteinized with trichloroacetic acid (sample/TCA: 1/1). The resulting contents were centrifuged (for 15 min 1500 × g). The same samples were used for zinc determination by using both atomic adsorption spectrophotometer (AAS) and enzymatic method[2,14].

RESULTS AND DISCUSSION

Trace heavy metals are dangerous in the environment due to their serious toxicity although presenting at very low concentrations. They may accumulate in the food chain. The development of new methods for quantifying trace metals is required and challenged. Most of the sensitive and selective methods recently available such as inductively coupled plasma mass spectrometry (ICP-MS), inductively-coupled plasma atomic emission spectrometer (ICP-AES) and graphite furnace atomic absorption spectrometry (GF-AAS) are too expensive and are not practically applied in developing countries[2].

It is critical for an analytical method to exhibit high sensitivity, selectivity and ease of usage in order to measure the concentration of trace metal ions in environmental science, life sciences, energy science and

materials science. Preparative procedures for the separation and concentration of the trace elements are usually required, because only trace amounts are present and other species in the same sample solution may interfere with the analysis of the element's composition.

In this study, we tried an alternative cost-effective method based on enzymatic principal for Zn^{2+} in milk and dairy products. To use enzymatic method, standard curve obtained with constant apoCA (3.6×10^{-5} M) and changing Zn^{2+} concentrations are presented in Fig. 1.

Table-1 shows the statistical analysis of the results of seven milk and dairy products. According to the Table-1, zinc levels in the (i) whole milk, (ii) skimed milk, (iii) cheese, (iv) yogurt, (v) ice cream (vanilla) (vi) butter) are normal value and agree with the literature. The zinc amount in milk and dairy products taken from Erzurum is acceptable.

TABLE-1
STATISTICAL EVALUATION OF THE RESULTS

Milk and dairy products	Zn^{2+} determination (mg/100 g) N:10		Student's t-test		Correlation	
	Enzymatic method	AAS method	t	P	r	P
Whole milk	0.3595 ± 0.007	0.3621 ± 0.016	0.287 >	0.05	0.995 <	0.05
Skimed milk	0.3598 ± 0.006	0.3564 ± 0.0071	0.623 >	0.05	0.987 <	0.05
Cheese	2.9826 ± 0.038	2.9431 ± 0.113	0.574 >	0.05	0.999 <	0.05
Yogurt	0.8809 ± 0.010	0.8793 ± 0.0063	0.225 >	0.05	0.998 <	0.05
Ice cream (Vanilla)	0.8862 ± 0.031	0.8832 ± 0.250	0.132 >	0.05	0.993 <	0.05
Butter	0.3267 ± 0.025	0.3200 ± 0.03	0.295 >	0.05	0.976 <	0.05

When the results of the AAS and enzymatic method was compared, it is easily seen from the r and p value (all of them < 0.05) that there is a highly positive correlation between the two methods. In addition, t-test results support that there was no statistical significant difference between the results found with two methods[17].

Furthermore, the stability of apo CA as a protein and the high esterase activity of bovine CA are important advantages of this method.

In conclusion, the enzymatic method can easily be applied in milk and dairy products zinc determination. This method is cheap and can be almost at any labrotory. On the other hand, studies on its application to auto analytic equipment and routine use are continuing.

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