An ion-based chromogenic method of detecting for inorganic phosphate in serum and milk

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ABSTRACT

A rapid method for the determination of inorganic phosphate in serum and milk by an ion-based chromogenic is described. Serum samples were detected directly by our system, and milk was also detected after degreased through centrifugation. By this procedure the samples are not diluted. Mean serum inorganic phosphate concentration found in healthy individual is 1.14mmol/L. Values found in serum is in good agreement with those previously reported. Mean inorganic phosphate concentration from foremilk and commercial milk are 2.5mmol/L and 12.5mmol/L respectively.

Keywords: UV-Vis Spectra; Pyrocatechol Violet; Ytterbium Chloride; Phosphate; Serum; Milk

1. INTRODUCTION

Phosphate is involved in important biomineralization processes such as bone formation and also processes that are clearly pathological such as the genesis of renal stones. Consequently, its determination in biological fluids is important [1]. In a clinical setting, inorganic phosphate levels in serum are determined as part of a routine blood analysis. The typical inorganic phosphate concentration in human serum range is 0.81-2.26mmol/L [2]. Individuals with abnormally high phosphate levels are diagnosed with hyperphosphatemia, which manifests in acute or chronic renal failure, hypoparathyroidism and excessive Vitamin D intake. And we know that higher serum phosphate levels would be associated with increased mortality risk among people with CKD [3-5]. Those with low inorganic phosphate levels suffer from hypophosphatemia which can be associated with rickets, hyperthyroidism, or Fanoci Syndrome [6-8]. In addition, there is generally a reciprocal relationship between serum calcium and inorganic phosphorus levels. High inorganic phosphorus in serum restrains the intake of calcium.

Regarding the newborn baby, foremilk or milk is the most main headspring they grow on. But exactly this time is the quickest time when young child grows, being in the bone blooming period and the cerebrum and the intelligence still being imperfect stage. So the right amount nutrition could guarantee the normal growth, and prevent malnutrition, rickets, anemia and so on. Especially if absorbing calcium phosphorus imbalance, can cause the low calcium blood sickness, the rickets [9]. Moreover iron in the milk is easy to form insolubly iron compound when affected by high phosphate and calcium, cannot be absorbed by the human body, which may cause the young child to occur lacking the iron anemia. In normal foremilk the calcium phosphorus proportion is 2: 1, is easy to be absorbed, to prevent and control the rickets. But the milk is 1: 2, is not easy to be absorbed. Therefore, determination the calcium phosphorus content from foremilk and milk is very important.

Most of the procedure for the colorimetric determination of inorganic phosphate are based on the formation of molybdophosphoric acid with further reduction to heteropolymetric molybdenum blue [10-12] on direct measurements of molybdo- and vanadomolybdophosphoric acid [13,14], or on complex formation between molybdophosphoric acid and basic dyes [15]. These chemical methods have serious shortcomings, however: Molybdate reduction is affected by slight changes in pH, the rate of complex formation is markedly influenced by protein concentration, and the acidity required leads to hydrolysis of organic phosphate, which results in over estimates of P_i concentration [16].

In our work, we developed a rapid method for the determination of phosphate in serum and milk by an ion-based chromogenic. Serum samples were detected directly by our system, and milk was also detected after degreased through centrifugation. By this procedure all samples are not diluted. Owing to our system's prominent advantages, it can serve as a hopeful substitute for

the molybdenum reagent.

2. MATERIAL AND METHODS

2.1. Reagents and Chemicals

The chemicals used were of analytical-reagent grade. PV (Pyrocatechol violet) was purchased from Shanghai and sodium monhydrogenphosphate was purchased from Beijing. Ytterbium oxide was a product of Rare Earth Graduate School of China. HEPES was purchased from Sigma. All solutions were made up with deionized water. HEPES buffer solutions were obtained by adding NaOH 0.1M solution into 10 mM aqueous HEPES using a Beckman Φ50 pH meter. Ytterbium chloride was prepared from ytterbium oxide and 37% hydrochloric acid. Ytterbium ion solution was prepared by dissolving ytterbium chloride in water. Serum samples were collected from health volunteers and stored at -17℃ until analyzed. Cow serum was purchased from commercial pured product. Human milk and commercial milk were degreased through a Centrifugal filter.

2.2. Instruments and Apparatus

pH determinations were performed using a Beckman Φ50 pH meter. UV-v(V)is spectra were recorded on a HP8453 spectrophotometer. PO-120 quartz cuvettes (10mm) were purchased from Shanghai city of China. Finnpipette Digitals were purchased from Shanghai of China. BFX5-320 Low Speed Automatic Balance Centrifuge was purchased from Baiyang Centrifuge factory. Olympus 2700 Complete Automatic Clinical Biochemistry Analysis Apparatus was purchased from Japan.

2.3. Measurement Procedure

Using the PV-HEPES-Yb^{3+} ensemble, we detected inorganic phosphorus in serum and milk samples. The procedures were as follows. In 10mM, pH 7.0 HEPES buffer containing 50μM PV and 100μM Yb^{3+} (a blue solution), the serum sample from one healthy volunteer was gradually titrated into the solution. At the same time the changes in the absorption peaks of solution in the UV–Vis spectrum were recorded. When no more changes in the absorption peaks of the system took place, titration came to a halt. Then we could calculate the inorganic phosphorus concentration in serum. Likewise, the inorganic phosphorus concentrations of human milk degreased or commercial milk were obtained by above detecting method.

3. RESULTS AND DISCUSSION

### 3.1. UV–Vis Spectra

Figure 1a shows the UV–v(V)is spectra obtained when
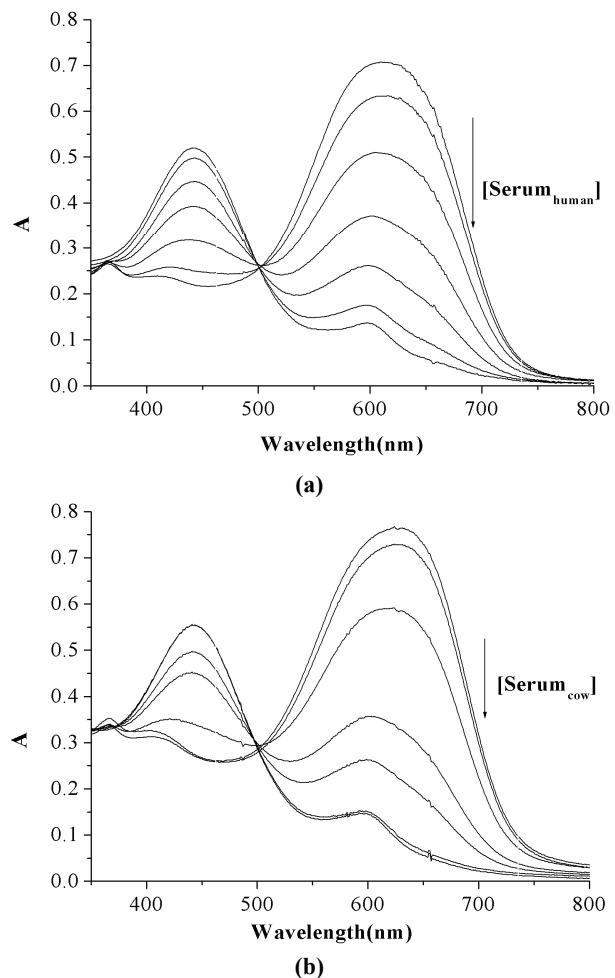
Figure 1. (a) The serum from human was added into PV-HEPES-Yb^{3+} with Vserum=0-215 μl; (b) the serum from cow was added into PV-HEPES-Yb^{3+} with Vserum=0-90 μl.

titrating the serum from a health human into the 10 mM, pH 7.0 HEPES buffer solution containing 100 μM YbCl$_3$ and 50 μM PV. With the addition of serum, the absorption peak at 623 nm decreased, while the peak at 444 nm increased. When the total volume of added serum reached 210 μL, titration ended. The concentration of inorganic phosphorus was 0.95 mmol/L. Similarly, **Figure 1b** shows UV–v(V)is spectra of serum from cow titrated. The concentration of inorganic phosphorus from cow was 2.20 mmol/L. **Figure 2a** shows UV–v(V)is spectra changes when titrating milk from healthy woman into our system. The inorganic phosphorus concentration of milk from lactation mother was 2.31 mmol/L. **Figure 2b** shows UV–v(V)is spectra changes of milk from commerce process titrated. The inorganic phosphorus concentration of milk from commerce was 11.54 mmol/L.

3.2. Selectivity over Other Constituents

In published paper, we addressed the selectivity of the system. We knew that the ensemble exhibited excellent

selectivity towards phosphate anions over other common anions, including Cl^-, SO_4^{2-}, CH_3COO^-, HCO_3^- and ClO_4^- [17]. Serum contains many other organic and inorganic compounds, such as creatinine, bilirubin, sugar, albumen, inorganic salts and transition ion besides the aforementioned ordinary anions. Do these compositions show some responsibility for the changes in the UV−v(V)is spectra and color? We took the HEPES buffer as a blank, and then added 200 μL serum into it. The result shows that there is no UV−v(V)is absorbance in the range of from 350 to 1,000 nm, suggesting that there are no other absorbance peaks coming from other compounds of the serum in the range of detection in the UV−v(V)is spectra (**Figure 3a**). Thus, we may conclude that the changes in the absorbance peak in this range resulted completely from the measurement processes and there was no cumulation or disturbance. To prove that the universal existence of anions in serum incurs no disturbance to exclude the possibility of interference from iron or other cations in the measurement, the following experiments were carried out. Firstly, as soon as the excessive 2 mM $YbCl_3$ was added into the 2 mM HPO_4^{2-} solution (V_{YbCl3}/V_{HPO42-}=1.02:1), precipitation occurred, a clear solution was gained through centrifugation and decantation processes; the solution (from 0 to 500 μL) was then added into 2 mL 10 mM HEPES buffer containing 50 μM PV and 100 μM Yb^{3+}, and no changes in absorption peak intensity and color were observed, i.e., no phosphate was detected in the solution, thus suggesting that the Yb^{3+} could completely remove HPO_4^{2-} from solution by forming sediment. Similarly, we added the excessive $YbCl_3$ into the collected serum samples whose content of HPO_4^{2-} was presumably quantitated with our methods. After the mixture had been treated in accordance with the aforementioned procedures, a great deal of the disposed serum sample(0–500 μL) was added into
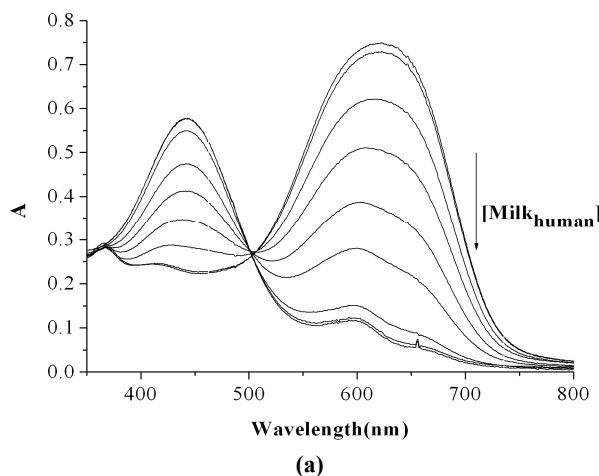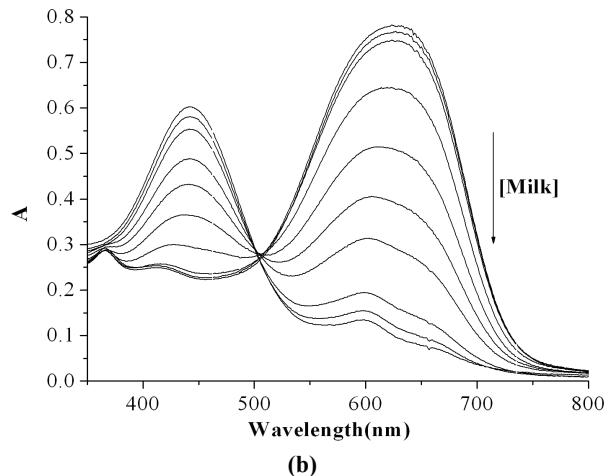

Figure 2. (a) The milk from woman was added into PV- HEPES-Yb^{3+} with Vmilk=0-65 μl; (b) the serum from cow was added into PV-HEPES-Yb^{3+} with Vmilk=0-13 μl.
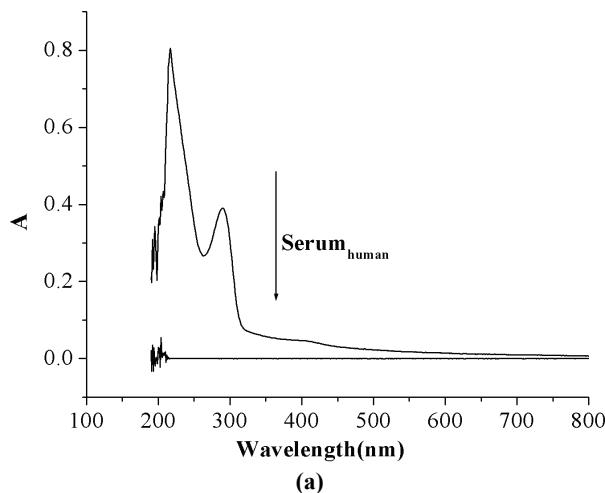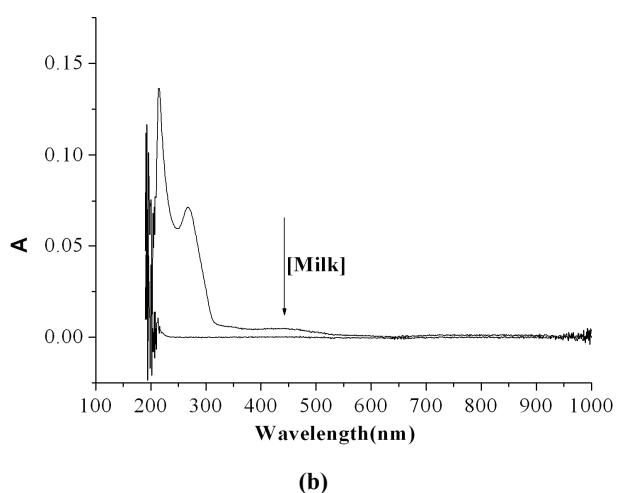
**Figure 3.** (a) UV/Vis spectra: Plot of absorbance (at λ=200-1000 nm) when adding 20 μl urine into HEPES buffer (pH 7.0); (b) when adding 50 μl milk into HEPES buffer (pH 7.0).
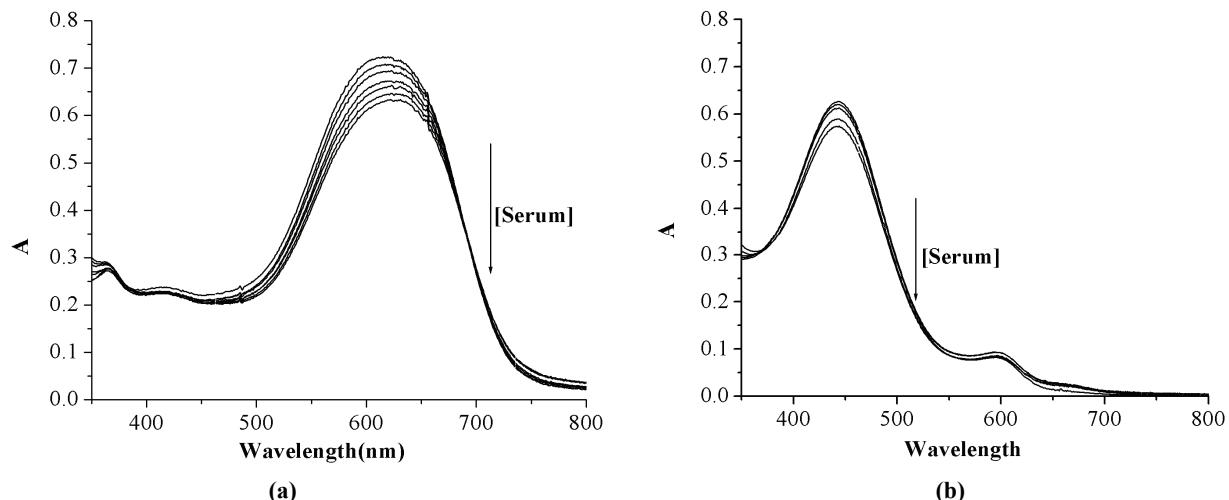
**Figure 4.** (a) the disposed serum sample (0-500 μl) was added into PV-HEPES-Yb^{3+}.(b) **UV/Vis** spectra （λ＝350～800nm）: no pretreatment serum sample (500 μl) was added into HEPES-PV (50 μM).

the 2 mL 10 mM HEPES buffer containing 50 μM PVand 100 μM Yb^{3+}, and no changes were observed in absorption peak intensity and system color(**Figure 4a**). The experiments excluded the possibility of interference from other anions with the measurement. In addition, we added an adequate amount of unpretreated serum sample into 2 mL 10 mM HEPES buffer only containing 50 μM PV, and no change in either the UV–v(V)is spectra or the system color was observed (**Figure 4b**). The system was still yellow. The experiments excluded the possibility of interference from iron or other cations with the measurement.

Now, we see about the selectivity of the detection system for milk. We took the HEPES buffer as a blank, and then added 50 μL milk into it. The result shows that there is no UV–v(V)is absorbance in the range of from 350 to 1,000 nm, suggesting that there are no other absorbance peaks coming from other compounds of the milk in the range of detection in the UV–v(V)is spectra (**Figure 3b**). Similary process was done for proving no disturbance from other composition of milk. All results ensure that our system is special to phosphate of milk.

3.3. Linearity and Detection Limits

Most instrumental methods available for the determination of phosphate in clinical samples have a common drawback; that is, their linear calibration range is too narrow. In our experiment, we plotted the curve with absorbance values at 623 nm against concentrations /0–2.5 mM/ of serum added to the PV-HEPES-Yb^{3+} system. We found our measurement obeyed the Beer–Lambert absorption law very well within the serum concentration range of 0–2.5mM. Linear regression with least-squares fitting yielded a correlation coefficient of
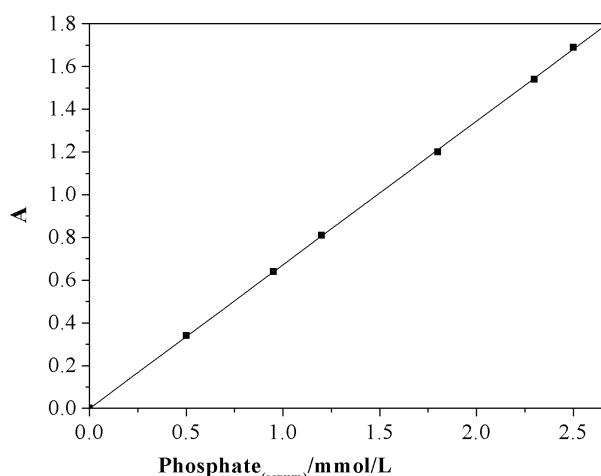

Figure 5. The working curve for serum measurement was plotted with the absorbance value against various concentrations of serum(0-2.5 mM)**.**

0.99995 (**Figure 5**). The lower detection limit of our method is around 10^{-4} M. And before, we have gotten our measurement obeyed the Beer–Lambert absorption law very well within the urine concentration range of 0–70mM [18]. Phosphorus concentrations of milk from woman or commerce are not higher than 70mM, so this linear calibration range is enough for milk.

3.4. Validation

In order to validate the accuracy of the method, we detected serum samples by the standard procedure (molybdenum blue assay for phosphate) and obtained equivalent results with our measurement. **Figure 6** and **Table 1** give the results (spectra) for serum obtained with the two kinds of detection methods. Finally, the recovery experiments were performed: The results are
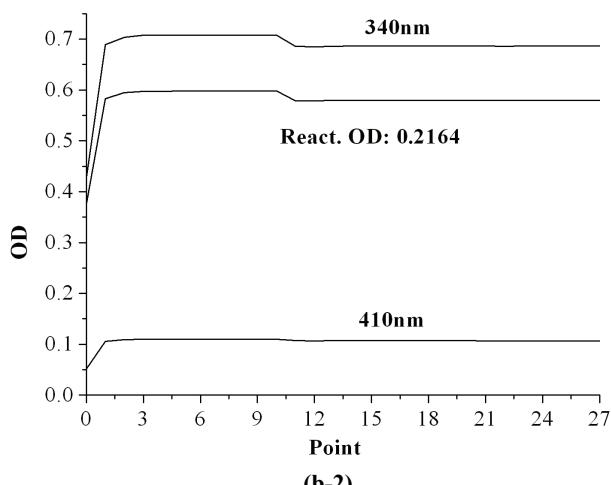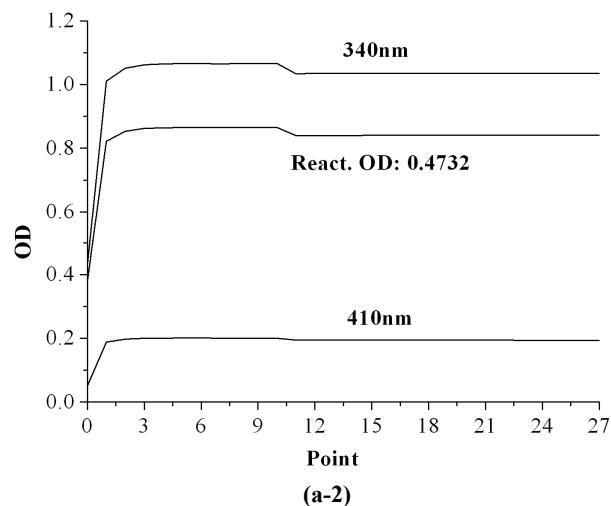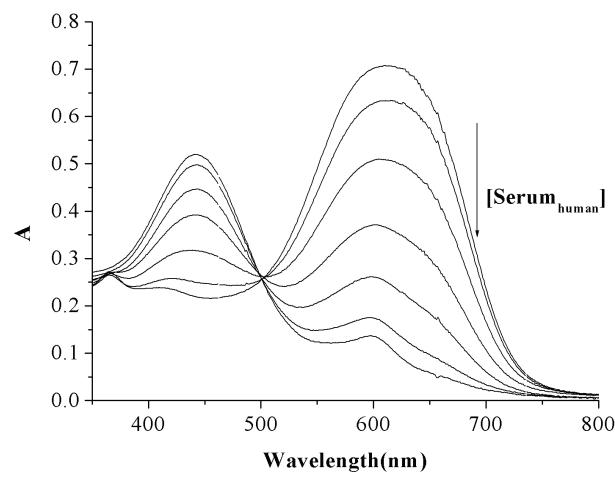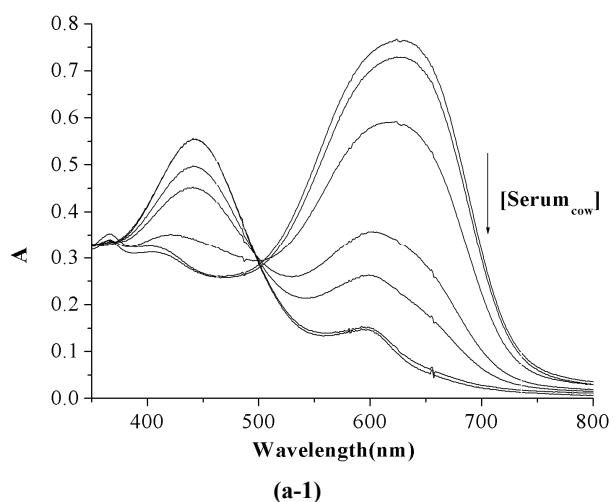
Figure 6. (a)Left: Uv-Vis spectra of cow serum sample Inorganic Phosphorus concentration from our method(2.20 mmol/L); Right: the results from Molybdenum blue assay for phosphate respectively(2.22 mmol/L; (b) Left: Uv-Vis spectra of serum sample Inorganic Phosphorus concentration from our method (0.95 mmol/L); Right: the results from Molybdenum blue assay for phosphate(0.93 mmol/L**).**

compiled in **Table 2**. The results indicated the accuracy of the method, as expressed by the calculated recovery values, was satisfactory.

3.5. Analysis of Results

From our data, we can see inorganic phosphorus content of milk is higher than foremilk about five times. This indicates the excessive inorganic phosphorus is disadvantageous to young child's growth. And with the people level of living enhancement, the commercial milk becomes the people basic nutriment. But in the processing commercial milk many important ingredients content are insufficient, for instance, the calcium, phosphorus ratio of is 2: 1 in milk containing the few calcium, many phosphorus, is easy to form the insoluble calcium phosphate, affects the intestinaltract absorbing calcium and

phosphorus. If provide turnips containing many calcium, few phosphorus for the child who eats the milk, can correct calcium and phosphorus proportion, namely can enhance the calcium absorbing capacity. Therefore, the reasonable increase and the adjustment can only prevent to be out of nutrition balance for one people drink milk.

3.6. Assay Advantage

Ion-based chromogenic method has proved to be a useful tool for clinical analysis because of its simplicity, repeatability, low reagent consumption and so on. We know that ion chromatography, the use of deproteinizing agents presents several disadvantages: perchloric acid and trichloroacetic acid need to be removed from the sample by time-consuming procedures otherwise they

Table 1. Results of our method against the method (molybdenum blue assay for phosphate).

Numbers of serum sample	Phosphate (mmolL^{-1}) from Molybdenum blue assay	Phosphate (mmolL^{-1}) from our method
1(Serum$_{human}$)	0.96	0.97
2(Serum$_{human}$)	0.93	0.95
2(Serum$_{cow}$)	2.20	2.22

Table 2. Recovery of phosphate in serum and milk.

Sample	Added (phosphate) mmolL^{-1}	Found (phosphate) mmolL^{-1}	Recovery (%)
1(Serum)	—	1.48	
	0.6	2.02	95.9
2(Serum)	—	1.11	
	0.6	1.73	102
3(Serum)	—	0.95	
	0.6	1.53	97.3
4(Milk$_{human}$)	—	2.34	
	1.0	3.42	104
5(Milk$_{cow}$)	—	12.5	
	5	17.3	98.4

interfere with the elution profile; organic solvents such as acetonitrile lead to damage to the column and sulphosalicylic acid is often contaminated with sulphate, lead to sample dilution. And our method decreases the cost of analyses with respect to batch methods involving enzymes in solution. All these advantages make the method reported here be a valid alternative for the determination of phosphate in serum.

4. CONCLUSIONS

To sum up, we developed a sensitive, rapid and direct method for detecting serum and milk phosphate spectrophotometrically. Our method is suitable for performing direct determinations of phosphate in serum without any pretreatment and any interference. Now more and more people are suffering from lithiasis as a result of better living standards. Timely inspection of serum phosphate is one of the clinical means of diagnosis. Since long-time, people only pay attention to the inorganic phosphorus determination in the urine and the blood serum, to determine the inorganic phosphorus in the foremilk mother's milk and milk method very little was mentioned. In this paper, we use our invention system to quantificationally determine inorganic phosphorus concentration from milk degreased through centrifugation, the results are accurate, suit to clinical and the commerce use.

5. ACKNOWLEDGEMENTS

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