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Simultaneous determination of vitamin B12 and cobalt (II) in infant milk power using liquid chromatography-inductively coupled plasma mass spectrometry

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A new method was developed for simultaneous determination of cobalamin compounds [cyanocobalamin (CN-Cbl), hydroxocobalamin (OH-Cbl), 5′-deoxyadenosylcobalamin (Ado-Cbl) and methylcobalamin (Me-Cbl)] and inorganic cobalt ions [Co(II)] in infant milk powder by liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICP-MS). The samples were enzymatic hydrolyzed by pepsin (porcine source) and the analytes extracted in the hydrolysate were measured directly by LC-ICP-MS after filtration. The chromatographic separation was performed on a Phenomenex Luna 5 µm C_{18} (250 mm × 4.6 mm) column. The mobile phase was consist of 1.6 mM ethylenediaminetetraacetic acid disodium salt (EDTA•2Na) and 0.4 mM potassium dihydrogen phosphate in 60% (v/v) methanol-water (pH = 6.0) (mobile phase A) and ultrapure water (mobile phase B). After LC separation with gradient elution, Co(II), OH-Cbl, CN-Cbl, Ado-Cbl, and Me-Cbl were measured as ^{59}Co by ICP-MS. The method detection limit of Co(II), OH-Cbl, CN-Cbl, Ado-Cbl, and Me-Cbl were 0.144 µg/kg, 0.197 µg/kg, 0.267 µg/kg, 0.209 µg/kg and 0.278 µg/kg, respectively. At three spiked levels (1.0, 10.0, 50.0 µg/kg), the average recoveries (%) of Co(II), OH-Cbl, CN-Cbl, Ado-Cbl, and Me-Cbl ranged from 70.4 to 79.6, 74.5 to 77.4, 91.0 to 93.1, 83.0 to 83.8, and 84.3 to 90.2, respectively. The relative standard deviations (RSD, %) of Co(II), OH-Cbl, CN-Cbl, Ado-Cbl, and Me-Cbl ranged from 0.50 to 2.57, 0.70 to 6.29, 0.80 to 2.37, 1.33 to 2.50, and 1.16 to 2.40, respectively. These results indicating that the method had a good accuracy and precision. The preparation of sample was simple and less time consuming since it avoiding solid phase extraction and follow steps.

Keywords Cobalamins, Vitamin B_{12}, LC-ICP-MS, Infant milk powder

Vitamin B_{12}, a tetrapyrrole complex containing cobalt ion, plays important role in metabolic processes and protein synthesis. There are four different forms of vitamin B_{12} (methylcobalamin, hydroxocobalamin, 5'-deoxyadenosylcobalamin and cyanocobalamin); among them, the most stable form is cyanocobalamin to which the other vitamers can be converted by reaction with cyanide[1]. Human body cannot synthesize cobalamin on their own and can only get it from animal derived foods such as meat, milk and algae[2]. Vitamin B_{12} plays an important role in nervous system development, erythrocyte production, fatty acids, and the synthesis of certain proteins. Infants, pregnant women and the elderly are at high risk of vitamin B_{12} deficiency, which can cause changes in the blood system and nervous system, affect the development of infants' cognitive ability, and increase the risk of pregnancy for women[3–7]. The assessment of vitamin B_{12} intake of infants is of high importance[8]. Vitamin B_{12} must be added to infant milk powder according to Chinese National Standard (GB 10765 – 2010) with concentration of 0.5–7.2 µg/100 g[9]. This is in consistent with the range of vitamin B_{12} prescribed by the CODEX STAN73-1981 Standard of the Codex Alimentarius Commission (CAC)[10]. CN-Cbl is less light sensitive and more stable than OH-Cbl, Me-Cbl and Ado-Cbl, so the added cobalamins in infant milk powder is synthetic CN-Cbl in most cases[11].

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High performance liquid chromatography (HPLC) and microbiological assay (MBA) are currently the most used methods for determination of vitamin B_{12} in foods. Detector used in HPLC method could be variable wavelength detector (VWD)[12,13], diode array detector (DAD)[14–16]or fluorescence detector (FLD)[1]. However, due to the low sensitivity of HPLC detector, purification of the sample and enrichment of the analytes are still needed by such as solid phase extraction to obtain a lower detection limit even in vitamin health products which have high vitamin B_{12} content []. The whole process is cumbersome with complex operation. For the determination of vitamin B_{12} in infant food and dairy products, both AOAC and China National Standard methods adopt a complex and time consuming MBA, but the method is not specific for the quantification of cobalamins[17–20]. In recent years, hyphenated techniques have been applied to vitamin B_{12} determination. Ma et al. concentrated vitamin B_{12} in fortified foods with solid phase extraction, and then determined it by ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with method detection limit (MDL) of 1 μg/kg[21]. Raju et al. extracted vitamin B_{12} from fortified breakfast cereals and multivitamin tablets by means of dissolution centrifuge and filtration, separate on a C_{18} column and measured with an ICP-MS[22]. The separation could be accomplished within 9 min with MDL of 0.9 μg/kg. Yang et al. extracted vitamin B_{12} from health products by microwave-assisted extraction, and measured by HPLC-ICP-MS with a similar column and separation time[23]. Liu et al. measured vitamin B_{12} in functional drink, multivitamin tablets and infant milk powder by solid phase extraction column followed with a size exclusion chromatography-inductively coupled plasma mass spectrometry (SEC-ICP-MS) with MDL of 0.4 μg/L[24]. A new method published in 2025 report a cyanide-free method using hydrophilic interaction liquid chromatography with fluorescence detection, but still has a high LOD of 0.4 μg/100 g of dry weight[1].

The unique chemical nature of cobalamins, characterized by a cobalt (Co) complex containing a corrin system made it can be measured by ICP-MS with very selective detection and high sensitivity[8]. Though CN-Cbl is the main form of cobalamins that added in the infant milk powder, the other forms of cobalamins as well as cobalt ions also needed to be separated and measured since ^{59}Co is the ion monitored in the ICP-MS. Otherwise, the other forms of cobalamins as well as cobalt ions may interfere with the quantification of CN-Cbl. Due to the development of the dietary supplement market and consumers' needs, an increasing number of new dietary supplement products contain "natural form vitamin B_{12}" or "high-bioavailable form vitamin B_{12}" (such as methylcobalamin) or a mixture of different cobalamins[14]. Similar trends may exist in infant milk powder market and a method that can measure OH-Cbl, CN-Cbl, Ado-Cbl, Me-Cbl and Co(II) simultaneously in infant milk powder is needed. Quantification of the accurate type of vitamin B_{12} added in infant milk powder may help with better supervision of the quality of infant milk products in China. But to the best of our knowledge, a method for simultaneous detection of multiple cobalamins in infant milk powder with high sensitivity and high extraction rate without complicated pre-treatment enrichment has not been reported yet.

Since direct extraction has long seen improvements for the targeted measurement of analytes[25]. In this paper, we optimized the conditions of pretreatment and liquid chromatography, four cobalamins (CN-Cbl, OH-Cbl, Me-Cbl and Ado-Cbl) and Co(II) were extracted from sample directly and separated simultaneously. Also, the conditions of ICP-MS were optimized to eliminate the interference of polyatomic molecules from mobile phase and/or milk powder matrix. Based on the advantages of high selectivity, low detection limit and wide linear range of LC-ICP-MS, a method for measurement of four cobalamins and Co(II) in infant milk powder samples was established, which can provide reliable technical support for quality supervision of infant milk powder as well as other dairy products.

Materials and methods

Reagents and standards

Ethylenediaminetetraacetic acid disodium salt (EDTA•2Na), acetic acid (99.9%) and pepsin (from porcine gastric mucosa, enzymatic activity ≥ 250 U/MG) were purchased from Anpel (Shanghai, China). Potassium dihydrogen phosphate (KH_2PO_4) (99.99%), ammonium acetate (≥ 99.0%) and ammonium hydroxide (28%) were purchased from Aladdin (Shanghai, China). Methanol (gradient grade) was obtained from Merck (Darmstadt, Germany). Ultrapure water was freshly produced from a Milli-Q® water purification system (Merck, Darmstadt, Germany). Cyanocobalamin (CN-Cbl) (pharmaceutical primary standard), hydroxocobalamin acetate (OH-Cbl) (certified reference material, pharmaceutical secondary standard), 5'-deoxyadenosylcobalamin (Ado-Cbl) (≥ 97.0%), and methylcobalamin (Me-Cbl) (pharmaceutical primary standard) were obtained from Merck (Darmstadt, Germany). Cobalt(II) standard solution (1000 mg/L) was purchased from National Analysis and Testing Center for Non-ferrous Metals and Electronic Materials (Beijing, China).

The extraction solution (19.3 g/L ammonium acetate solution) was prepared by dissolving 19.3 g ammonium acetate in 1000 mL ultrapure water and the pH was adjusted to 4.0 by acetic acid. Bovine-milk-based infant milk powder sample was purchased from a local market in Hangzhou (Zhejiang Province, China) and kept in dark place. The stock solutions of CN-Cbl, OH-Cbl, Me-Cbl and Ado-Cbl and Co(II) was prepared at a concentration of 1.0 mg/L in extrction solution. All stock solutions were stored in a refrigerator at −20 ℃. Calibration standards, ranging from 0.10 to 10.0 μg/L, were prepared with the extraction solution and stored at 4 ℃ within 2 weeks. The whole process of standard preparation should be carried out in a dark room equipped with red lights to avoid possible conversion of the cobalamins.

Instrument

An ULC 510 LC system (EXPEC technology, Hangzhou, China) consisted of a quaternary pump and an autosampler was used for chromatographic separation. Separation of four cobalamins and Co(II) was accomplished by using a Phenomenex Luna C_{18} column (5 μm, 250 mm × 4.6 mm). The mobile phase A was 1.6 mM EDTA·2Na and 0.4 mM KH_2PO_4 in 60% (v/v) methanol-water (pH = 6.0), the mobile phase B was ultrapure water. Gradient elution was used for separation, as shown in Table 1. The column outlet was connected to the nebulizer of the ICP-MS device through a polyetheretherketone (PEEK) adapter.

A SUPEC 7000 ICP-MS (EXPEC Technology, Hangzhou, China), equipped with TEC refrigeration module which can maintain atomizing chamber temperature at 0 °C, was used for the determination of cobalt. Daily tuning is performed using a tuning solution which contains 40% methanol. The optimization of the oxygen flow rate was operated as follows: First, the oxygen flow rate was set to 50 mL/min to prevent carbon deposition. Second, reduce the oxygen flow rate slowly until the core of the plasma appears green. Third, increase the oxygen flow rate until the green just disappeared. And this flow rate of oxygen is the best oxygen flow rate (20 mL/min) and the plasma is stable. It can burn out organic matter efficiently, decrease the deposition of the carbon and have no damage to the cone. Instrument control and data processing were carried out by means of MassAnalyst (Version: MassAnalyst.P004.V01A.002, EXPEC Technology, Hangzhou, China). The LC-ICP-MS parameters were listed in Table 1.

Sample preparation

Approximately 5.00 g of infant milk powder was weighed into a 50 mL polyethylene centrifuge tube (wrapped with tinfoil), and 25 mL of extraction solution and 0.04 g pepsin were added into the tube successively. After mixing gently, the milk powder was hydrolyzed in a 37 °C water bath thermostatic oscillator for 0.5 h. Afterwards, the tube was transferred to a 100 °C water bath and kept for 0.5 h to stop the hydrolysis. After cooling to room temperature, the tube was centrifuged at 10,304 g for 8 min. Finally, approximately 1.5 mL of the supernatant were filtered through a 0.45 µm filter and the filtrate was collected in a 2 mL brown autosampler vial. Similar to standards preparation, the whole process of sample preparation should be carried out in a dark room equipped with red lights and all the tubes were wrapped with tinfoil.

Method evaluation

To evaluate the linearity, standards of Co(II), OH-Cbl, CN-Cbl, Ado-Cbl and Me-Cbl were prepared for the following concentrations i.e., 0.00 µg/L, 0.10 µg/L, 0.50 µg/L, 1.00 µg/L, 5.00 µg/L, 10.0 µg/L with the extraction solution. Calibration curves were obtained from the peak area of each compound versus the corresponding concentration using linear regression. For each batch of analysis, working standard solutions were prepared by stepwise dilution of individual stock solutions before use. The method was validated according to the National Standard of China "GB/T 27404 – 2008 Criterion on quality control of laboratories-Chemical testing of food"[26]. The lowest point of the standard curve, i.e. 0.10 µg/L of mixed standard solution, was measured 7 times and the instrumental detection limit (IDL) were calculated as 3 times of the standard deviation (SD), and the instrumental quantification limit (IQL) was defined as 3 times of the IDL. Microsoft Excel (Microsoft Office

LC conditions			
Analytical column	Phenomenex Luna C_{18}, 5 µm, 250 mm × 4.6 mm		
Mobile phase A	1.6 mM EDTA·2Na and 0.04 mM KH_2PO_4 with 60% (v/v) Methanol-H_2O (pH = 6.0)		
Mobile phase B	Ultrapure water		
Column temperature	Room temperature		
Injection volume (µL)	100		
Flow rate (mL/min)	1.0		
Gradient Program	Time (min)	A (%)	B (%)
	0.00	20	80
	8.00	70	30
	15.00	20	80
ICP-MS conditions			
Nebulizer gas flow rate (L/min)	0.770		
Auxiliary gas flow rate (L/min)	1.00		
Plasma gas flow rate (L/min)	15		
Oxygen flow rate (mL/min)	20.00		
RF power (W)	1600		
Acquisition mode	Peak hopping		
Dwell time (ms)	10		
Atomizing Chamber temperature (°C)	0		
Sample/skimmer Cone	Platinum cones		
Central tube (mm)	1.0		
Ion monitored	^{59}Co		
Mode	He collision		

Table 1. Operating conditions of LC-ICP-MS.

Home & Student 2021 for Windows, version 2406, Redmond, WA) was used for calculations and statistical analysis of the validation data. The method detection limit (MDL) and method quantification limit (MQL) were calculated with a sample volume and dilution factor (commonly sample mass was 5.00 g and dilution factor was five) based on IDL and IQL (Eqs. 1, 2). To validate the recovery and precision of this method, an infant milk powder purchased from a local market was randomly selected. Then, six separate aliquots of the infant milk powder (approximately 5.00 g for each aliquot) were spiked at three different levels (i.e. 1.0, 10.0, and 50.0 μg/kg). The recovery was expressed as the percentage of the expected values[26], and the precision (within-day) was expressed as the relative standard of deviation (RSD, %). The samples at spiking level of 10.0 μg/kg were remeasured 1 day, 2 days and 4 days after spiking to investigate the stability of the sample which stored at room temperature and keep away from direct sunshine. Moreover, for the purpose of evaluating the carryover effect of the high-concentration sample to the next sample in the analysis sequence, three blank sample (i.e. ultrapure water) were alternately injected immediately after each standard solution with the highest concentration. To avoid the possible conversion of any cobalamins, the whole pretreatment steps were carried out in a dark room.

$$MDL = \frac{IDL \times V}{m} \tag{1}$$

$$MQL = \frac{IQL \times V}{m} \tag{2}$$

MDL (μg/kg): method detection limit; *IDL* (μg/L): instrumental detection limit; *MQL* (μg/kg): method quantification limit; *IQL* (μg/L): instrumental quantification limit; *V* (mL): volume of extraction solution; *m* (g): weight of sample.

$$R = \frac{m_1 - m_0}{m} \times 100\% \tag{3}$$

R (%): Recovery of the method; m_1 (μg): amount of analyte measured in the spiked milk powder sample; m_0 (μg): amount of analyte measured in the original milk powder sample; *m* (μg): amount of analyte spiked in the original milk powder sample;

Comparison of different enzyme

A triplicate enzymatic efficiency study was designed with protease type as the sole variable. Three parallel-treated sample sets (5.00 g each) were respectively digested with: (a) pepsin (porcine source), (b) trypsin, and (c) pepsin, following the standardized hydrolysis protocol (Sect. 2.3).

Results and discussion

Optimization of LC operating conditions

The separation of four cobalamins and Co(II) was carried out on a reversed phase C_{18} column. The chromatographic behavior of four cobalamins and Co(II) changed with different salt concentration of the mobile phase. Potassium dihydrogen phosphate (KH_2PO_4) solution is a commonly used mobile phases for separating various elements[27–29]. In the current study, separation with different concentrations of KH_2PO_4 solution [(a) 0.4 mM, (b) 0.8 mM and (c) 1.2 mM] were investigated. As shown in Fig. 1, the retention time of OH-Cbl, CN-Cbl, Me-Cbl and Ado-Cbl increased significantly while the concentration of KH_2PO_4 increasing. The five analytes [Co(II), OH-Cbl, CN-Cbl, Me-Cbl and Ado-Cbl] both showed good separation when the concentration of KH_2PO_4 solution was 0.4 mM and 0.8 mM, and the total run time was slightly shorter when the concentration of KH_2PO_4 solution was 0.4 mM. When the concentration of KH_2PO_4 solution increased to 1.2 mM, the peak of OH-Cbl tailed heavily. Finally, 0.4 mM KH_2PO_4 was selected in the following experiments.

Adding methanol in the mobile phase can improve the peak shape as well, and shorten the retention time so as to shorten the analysis time. Considering that high percentage of methanol will increase the load of ICP-MS, 60% (v/v) methanol in mobile phase A is selected. The pH of the mobile phase may also affect the separation. We investigated the effect of different pH of the mobile phase. And we found that the retention time of all the five analytes did not change much, but the peak pattern of OH-Cbl was obviously improved and a better sensitivity could be obtained when the pH of the mobile phase A was 6.0. The addition of EDTA can also improve the separation effect[22]. However, when using the literature-reported concentration of 20 mM, we observed interference from unknown impurity peaks following the cobalt ion peak. We therefore systematically evaluated lower EDTA concentrations (1.6, 2.0, 3.0, and 5.0 mmol/L) to eliminate these artifacts. The results demonstrated complete elimination of impurity peaks at 1.6 mM EDTA, while maintaining adequate resolution for all five target analytes. Consequently, 1.6 mmol/L was selected as the optimal concentration. Hence, the mobile phase A was made up of 1.6 mM ethylenediaminetetraacetic acid disodium salt (EDTA•2Na) and 0.4 mM potassium dihydrogen phosphate in 60% (v/v) methanol-water (pH = 6.0).

Ultrapure water was used as mobile phase B to dilute mobile phase A and to achieve a good separation with a gradient elution. As shown in Fig. 2a, the elution procedure was initiated with 10% of mobile phase A and slowly increased to 100% within 8 min, then dropped back to 10% within 2 min. It was shown that the CN-Cbl and Ado-Cbl were partially overlapped and could not be separated thoroughly. Then, the elution procedure was optimized with initiated proportion of mobile phase A of 20% and slowly increased to 70% within 8 min, and finally dropped back to 20% within 7 min. With this procedure, the four cobalamins and Co(II) could be completely separated (Fig. 2b). These results demonstrate the chromatographic complexity of cobalamin compounds. Both the initial concentration and gradient steepness were found to significantly impact peak shape and resolution. This behavior likely stems from the compounds' large molecular weights, multiple active functional groups,

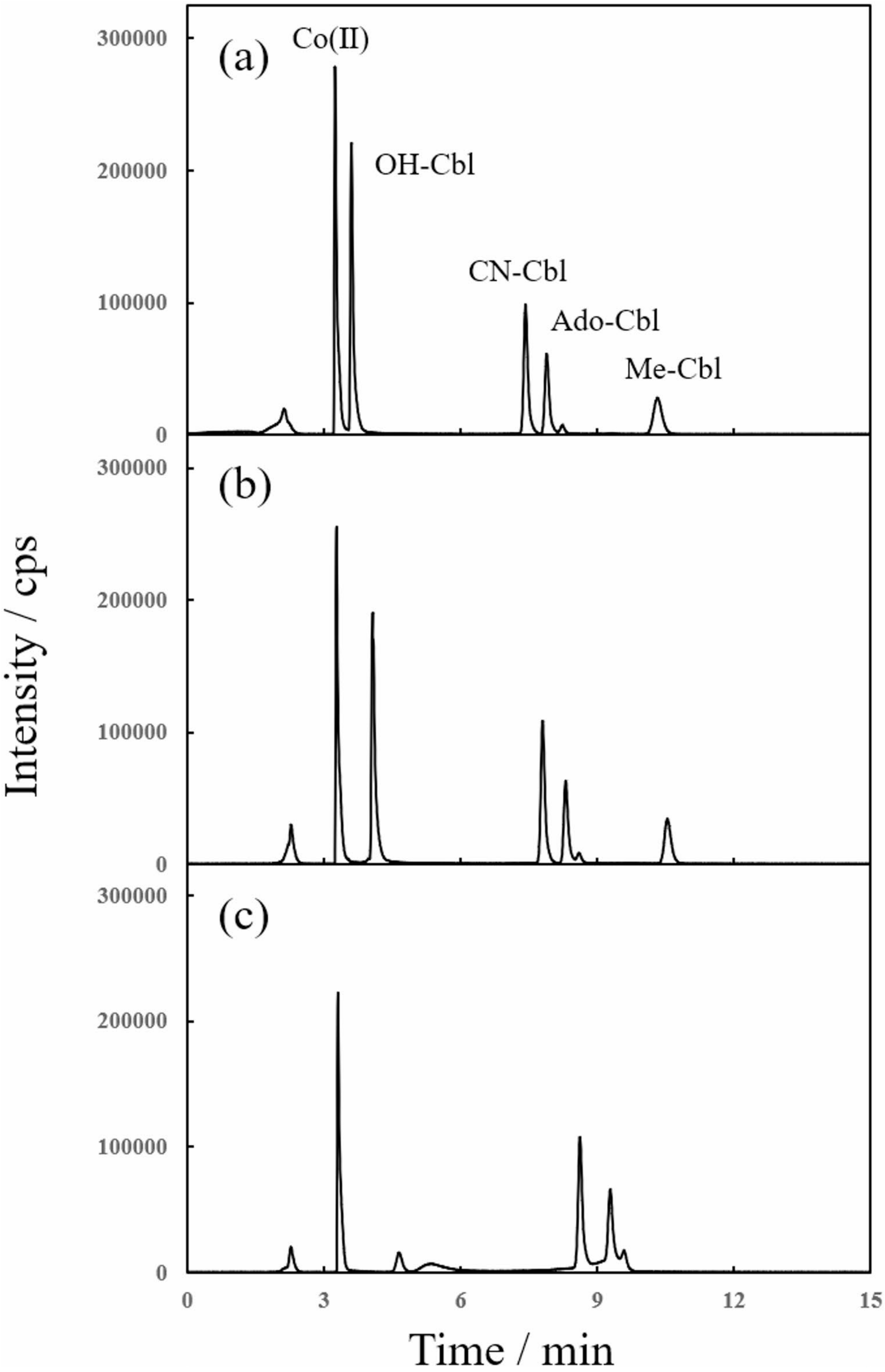

Fig. 1. Effect of KH_2PO_4 concentration in mobile phase A on chromatographic performance: (**a**) 0.4 mM (**b**) 0.8 mM and (**c**) 1.2 mM. Each cobalamin and Co(II) standard were present at 10 µg Co L^{-1}. Concentration of CH_3OH was 60% (v/v).

and intricate three-dimensional structures, which collectively render their separation susceptible to various chromatographic parameters. Consequently, meticulous optimization of separation conditions is essential to achieve satisfactory resolution. Further, there was a peak between Co(II) and OH-Cbl when the extract solution was used to prepare the standard solution (Fig. 2c). However, this unknown peak was well separated with OH-Cbl and did not affect the quantification of four cobalamins and Co(II).

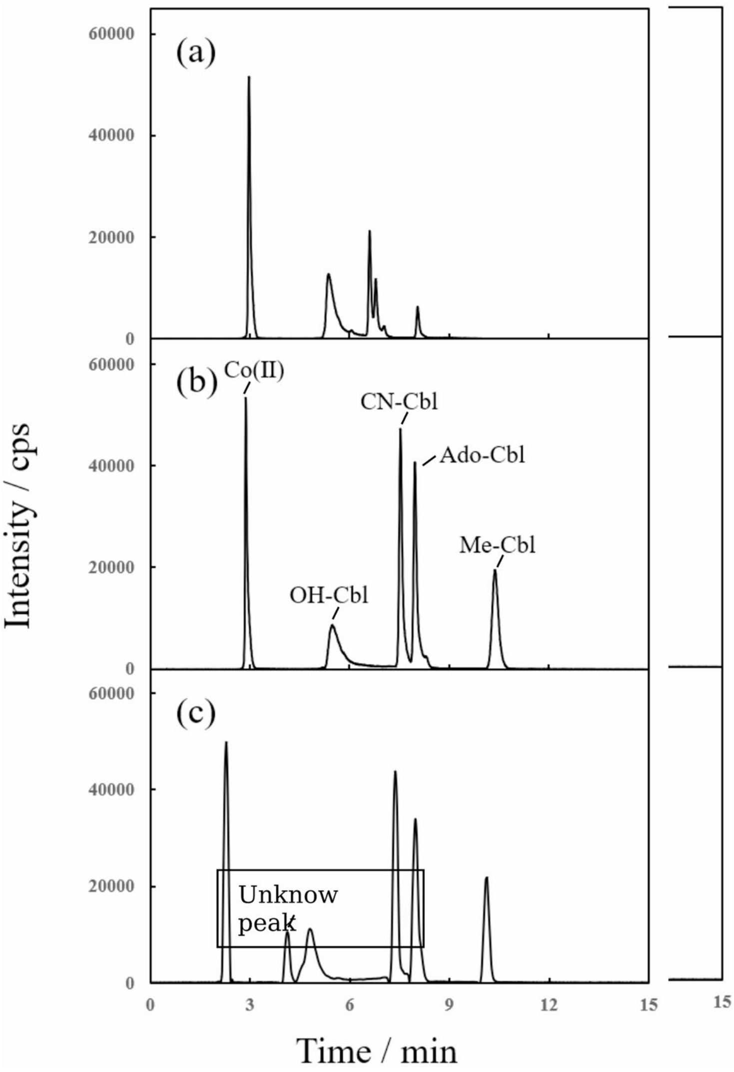

Fig. 2. Chromatograms of mixed standard solution with concentration of 2 μg Co L^{-1} for each analyte at different gradient procedure: (**a**) initiated with 10% of mobile phase A, the standard was prepared with water; (**b**) initiated with 20% of mobile phase A, the standard was prepared with water; (**c**) initiated with 20% of mobile phase A, the standard was prepared with extract solution.

In conclusion, the optimized mobile phase A was comprised of 0.4 mM KH_2PO_4, 1.6 mM EDTA and 60% (v/v) methanol (pH = 6.0), mobile phase B was ultrapure water, and an optimized gradient elution was used for separation (Table 1).

Optimization of ICP-MS operating conditions

Excessive organic load could reduce the intensity of the signal and the stability of the plasma, and may even extinguish the plasma when mobile phase enters the plasma torch[30] because the content of organic matter (methanol, EDTA, etc.) in the mobile phase can reach up to more than 42%. Carbon produced by the decomposition of methanol, EDTA and sample matrix in plasma will deposit on the cone surface, which will reduce the signal and increase the noise of the instrument, and even block the cone hole[8]. Wu et al. used a membrane desolvation system (Aridus II) to solve this problem, but this needs an extra instrument[30]. In this paper, three measures were taken to reduce the deposition of the carbon: (i) Instead of the classical 2.5 mm torch, a narrower 1.0 mm plasma torch was used to maintain the stability of the plasma and reduce sample volume; (ii) A TEC refrigeration device was used to control the temperature of atomizing chamber at 0 °C to reduce the methanol saturated vapor pressure and to prevent plasma quenching when the high proportion of methanol entered the plasma torch; (iii) Oxygen was used online to react with organic matter so to reduce the carbon deposition in the tube and cone hole, and the sensitivity of the signal and the stability of the plasma were improved. But too much use of oxygen will increase the load of ICP, consume too much energy of ICP to decompose oxygen, and eventually lead to decreased signal and plasma stability, and may cause damage to the sample cone. So, it is necessary to optimize the amount of oxygen added. A tuning solution prepared with 40%

(v/v) methanol which matched the mobile phase condition was used for daily tuning to adjust the amount of oxygen added.

Infant milk powder is a complex sample of matrix, which contains large amount of organic matter and is rich in Ca, Mg, O, H, N and other elements. These will cause massive spectrum interference of polyatomic molecules such as $^{43}Ca^{16}O^+$, $^{42}Ca^{16}O^1H^+$, $^{24}Mg^{35}Cl^+$, $^{36}Ar^{23}Na^+$, $^{40}Ar^{18}O^1H^+$, $^{40}Ar^{19}F^+$, which will affect the determination of ^{59}Co. The use of helium in collision mode enables collisional dissociation of the polyatomic ions, effectively suppressing detection interference. The baseline noise under standard mode was about 20 times higher than that under He collision mode. Therefore, collision mode is select to reduce the interference of the polyatomic molecules.

Optimization of sample preparation

Cobalamins are sensitive to light, pH and heat, though CN-Cbl is more stable, and may convert to other kinds of cobalamins/compounds during sample preparation. Potassium cyanide or sodium cyanide is usually used to convert OH-Cbl, Ado-Cbl and Me-Cbl into CN-Cbl, which is then purified and concentrated by solid phase extraction column for testing. But the use of cyanide in the laboratory is considered undesirable because of health and safety issues[1]. Microbial method was also used by measuring the correlation between the growth of *Lactobacillus Leichmannii* and vitamin B_{12} content. Both methods were complex, time-consuming, and less sensitive, and could not quantify each compound of cobalamins. Though determination of cobalamins in various samples, such as pharmaceutical preparations[31], cereal and multivitamin tablets[22], drinks[32] were reported, but reports on infant milk powder were rarely. The extraction and purification of each cobalamin compounds as well as Co ions from milk powder is a key point of the method.

Cobalamins in infant milk powder may exists both in a free form or bound to protein. Thus, the part that bound to protein should be released and then could be extracted. To quantify each individual cobalamin compound, a gentle method that could keep each cobalamin compound stable should be chose. In this study, ammonium acetate solution ($pH = 4.0$) was used as the buffer for enzymatic hydrolysis and extraction. We found that the pH of ammonium acetate solution was an important factor affecting the symmetry of chromatographic peaks as well as recovery of four cobalamins compounds and Co(II). The effect of different pH of ammonium acetate solution (i.e. pH 3.0, 4.0, 5.0 and 6.0) was investigated. With the decreasing pH of ammonium acetate solution, the retention time of OH-Cbl was shortened, the symmetry of the peak was improved, and the recovery of OH-Cbl was significantly increased. However, there is an unknown peak between Co(II) and OH-Cbl, and its peak height increased with the decreasing of pH. This unknown peak overlapped with OH-Cbl when the pH decreased to 3.0. Thus, ammonium acetate solution of pH 4.0 was selected as the extraction solution to prevent the interference of the unknown peak.

It is important to choose an appropriate enzyme for the enzymatic hydrolysis process because enzyme may lead to cobalamin's conversion. The effect of using pepsin (porcine source), trypsin and papain was investigated. Results showed that OH-Cbl and CN-Cbl both converted to unknown compounds with high conversion rate when papain was used. Little conversion was observed of all the four cobalamin compounds when using trypsin or pepsin (porcine source), but the recovery was slightly poor when using trypsin. So, pepsin (porcine source) was selected for the enzymatic process.

It is necessary to remove the protein dissolved in the extraction solution as much as possible for too much protein dissolved in the extraction solution may contaminate LC column and cause carbon accumulation in the cone. Salting out or organic solvents are commonly used to precipitate proteins. In this study, the effect of methanol (60%, v/v), potassium ferrocyanide solution (106 g/L) and zinc acetate solution (220 g/L), sodium acetate solution (20.5 g/L) as protein precipitator were investigated. Methanol (60%, v/v) could not precipitate the protein effectively, and the solution was still turbid even after high-speed centrifugation or filtered by 0.45 μm membrane. Potassium ferrocyanide solution (106 g/L) and zinc acetate solution (220 g/L) showed a good precipitation effect but the peak of OH-Cbl and Ado-Cbl disappeared after chromatographic separation. Similarly, the peak of Co(II) disappeared when using sodium acetate solution (20.5 g/L) as protein precipitator. In addition to the use of protein precipitant, the ultrafiltration centrifuge tube was also tried to remove proteins. However, due to the different material structure of the filter screen of the ultrafiltration centrifuge tube, only proteins larger than a certain molecular weight could be isolated, while other ions smaller than the molecular weight could not be guaranteed to be filtered, so the purpose of protein removal could not be achieved by using the ultrafiltration centrifuge tube.

Finally, we used a simple and easy method to remove the protein. After hydrolyzed with pepsin (porcine source), the proteins were denatured by boiling water bath for 30 min and then separated by centrifugation. With this method, Co(II) and the four cobalamins could keep stable and all analytes achieved a good recovery (Table 4) without any influence of the symmetry of the peak.

Method performance

The linear range of this method was 0–10.0 μg/L for all the analytes with coefficient correlation higher than 0.999. The IDLs of four cobalamins and Co(II) ranged from 0.029 to 0.056 μg/L, which was better than the reported HPLC-ICP-MS method of 0.04–0.08 μg/kg[22]. Base on the IDLs and IQLs, the MDLs ranged from 0.144 to 0.278 μg/kg and the MQLs ranged from 0.480 to 0.927 μg/kg (Table 2). The MDLs and MQLs of the current method can meet the requirements of vitamin B_{12} supervision of infant milk powder in China (0.5–7.2 μg/100 g)[9]. Compared with other methods, such as HPLC-UV[31,32], HPLC-ICP-OES[32], HPLC-DAD[14], HPLC[13], CZE-ICP-MS[23] and CE-ICP-MS[33], the current method has a much lower detection limit (Table 3).

Since most of the vitamin B_{12} in commercially available infant milk powder is artificially added cyanocobalamin, and there is no Standard Reference Materials with values for all four vitamers of vitamin B_{12} available, recovery was used to indicate the accuracy of the method. As showed in Table 4, at three spiked levels

Analyte	Linear Equations	R^2	IDL (μg/L)	IQL (μg/L)	MDL (μg/kg)	MQL (μg/kg)
Co(II)	y = 21276x + 14,749	0.9998	0.029	0.087	0.144	0.480
OH-Cbl	y = 14327x − 198	1.0000	0.039	0.117	0.197	0.655
CN-Cbl	y = 16750x − 203	0.9999	0.053	0.159	0.267	0.890
Ado-Cbl	y = 12809x − 152	0.9999	0.042	0.126	0.209	0.698
Me-Cbl	y = 15807x − 144	0.9997	0.056	0.168	0.278	0.927

Table 2. Linear equations, correlation coefficients (R^2), IDL, IQL, MDL and MQL data for four cobalamins and Co(II).

System	Kinds of cobalamins	IDL	MDL	Matrix	Reference
LC-ICP-MS	Co(II)	0.029 μg/L	0.144 μg/kg	Infant milk powder	Current method
	OH-Cbl	0.039 μg/L	0.197 μg/kg		
	CN-Cbl	0.053 μg/L	0.267 μg/kg		
	Ado-Cbl	0.042 μg/L	0.209 μg/kg		
	Me-Cbl	0.056 μg/L	0.278 μg/kg		
HPLC-UV[a]	CN-Cbl		0.16 mg/kg	Kefir samples	[32]
HPLC-ICP-OES[b]	Co(II)		0.06 mg/kg	Kefir samples	[32]
	CN-Cbl		0.07 mg/kg		
HPLC-VWD[c]	CN-Cbl		0.04 mg/kg	Supplemented foods	[13]
HPLC-DAD[d]	CN-Cbl		0.52 mg/L	dietary supplements	[14]
HILIC-FLD[e]	Total VB_{12}		4 μg/kg	Infant formula	[1]
HPLC-ICP-MS[f]	Co(II)	0.08 ng/g		Fortified breakfast cereals and multivitamin tablets	[22]
	CN-Cbl	0.04 ng/g			
CE-ICP-MS[g]	Co(II)	0.3 ng/mL		Nutritive supplements and chlorella Foods	[33]
	OH-Cbl	0.2 ng/mL			
	CN-Cbl	1.7 ng/mL			

Table 3. Comparison of methods for determination of cobalamins in the literatures. [a]HPLC-UV: High performance liquid chromatography-UV detection. [b]HPLC-ICP-OES: High performance liquid chromatography-inductively coupled plasma-optical emission spectrometry. [c]HPLC-VWD: High performance liquid chromatography- variable wavelength detector. [d]HPLC-DAD: High performance liquid chromatography-diode array detection. [e]HILIC-FLD: Hydrophilic interaction liquid chromatography-fluorescence detection. [f]HPLC-ICP-MS: High performance liquid chromatography-inductively coupled plasma mass spectrometry. [g]CE-ICP-MS: Capillary electrophoresis-inductively coupled plasma-mass spectrometry.

Analyte	Original value (μg/kg)	Spiking value (μg/kg)	Measured ± SD (μg/kg)	Average recovery (%)	RSD (%)
Co(II)	14.7	1.0	12.5 ± 0.30	79.6	2.41
		10.0	18.2 ± 0.47	73.8	2.57
		50.0	49.9 ± 0.23	70.4	0.50
OH-Cbl	< MDL	1.0	0.772 ± 0.048	77.0	6.29
		10.0	7.45 ± 0.23	74.5	3.10
		50.0	38.7 ± 0.26	77.4	0.70
CN-Cbl	2.31	1.0	3.22 ± 0.027	93.1	2.37
		10.0	11.2 ± 0.11	91.0	1.00
		50.0	48.2 ± 0.41	91.8	0.80
Ado-Cbl	< MDL	1.0	0.830 ± 0.12	83.0	2.29
		10.0	8.38 ± 0.11	83.8	1.33
		50.0	41.8 ± 1.04	83.5	2.50
Me-Cbl	< MDL	1.0	0.847 ± 0.010	84.7	1.16
		10.0	8.43 ± 0.13	84.3	1.60
		50.0	45.10 ± 1.06	90.2	2.40

Table 4. Recovery and precision of the method for each analyte ($n = 6$).

Sample	Total cobalamins[a]	OH-Cbl[b]	CN-Cbl[b]	Ado-Cbl[b]	Me-Cbl[b]	sum[c]
Infant formula 1	39.7	6.5	22.1	4.2	7.1	39.9
Infant formula 2	41.2	6.2	24.3	2.8	7.5	40.8
Infant formula 3	46.8	6.8	27.0	4.6	8.4	46.8
Infant formula 4	41.1	5.9	25.4	4.0	6.2	41.5
Infant formula 5	40.9	6.8	24.1	3.2	7.9	42
Infant formula 6	40.3	5.9	22.3	4.0	7.6	39.8
Infant formula 7	41.2	5.9	23.5	4.1	7.6	41.1
Regular whole milk powder	16.7	2.1	8.2	1.6	4.2	16.1

Table 5. Concentration of cobalamins in milk powder samples (µg/kg). [a]: Total cobalamins measured with National Food Safety Standard - Determination of Vitamin B_{12} in Foods[12]. . [b]Measured with current method. [c]Sum of each cobalamin measured with current method.

(1.0, 10.0, 50.0 µg/kg), the average recoveries (%) of each analyte ranged from 70.4 to 91.8%, and the relative standard deviations (RSD, %) of each analyte ranged from 0.50 to 6.29%. Meanwhile, considering that conversion may take place during the sample preparation, especially OH-Cbl, we also calculated the total recovery and total RSD [calculated by the sum of the four analytes except for Co(II)] of the method. The total recovery was 82.7% with total RSD of 0.78% (at spiking level of 10 µg/kg, $n = 6$), confirming that the method had good accuracy and precision further.

To investigate the stability of samples that stored at room temperature and keep away from direct sunshine, spiked samples (at spiking level of 10 µg/kg, $n = 6$) were remeasured 1 day, 2 days and 4 days after spike. Results showed that all the analytes could keep stable with average recovery ranged from 72 to 92% with RSD less than 5%, indicating that all the analytes could keep stable within 4 days at the current storage conditions. Carryover tests indicated that there was no cobalamin or Co(II) residues left and had no effect on the analysis of the subsequent samples.

Concentration of cobalamins in milk powder samples

For method validation, eight market-acquired milk powder samples (7 infant formula + 1 whole milk powder) were analyzed, demonstrating successful detection of all four cobalamin variants at concentrations of 2.8–27.0 µg/kg (Table 5). Meanwhile, we also analyzed these eight samples using the national standard method[12], and the results demonstrated high consistency between the two methods.

Limitations

Although the current method is sensitive and accurate, and the sample treatment is easy and less time consuming. There are still some limitations need to be mentioned. First, we only use one randomly selected infant milk powder to validate the method, though eight milk powder products were used for method application, this might not thoroughly represent all the other infant milk powder. Less effective of LC separation or worse accuracy and precision may occur when applied to other brand of infant milk powder. Second, a new conversion product of the four cobalamins (OH-Cbl, CN-Cbl, Me-Cbl and Ado-Cbl) during enzymatic hydrolysis was observed, but the source and factors which can affect this conversion process was still unclear. Further investigation of the conversion of the four cobalamins (OH-Cbl, CN-Cbl, Me-Cbl and Ado-Cbl) during enzymatic hydrolysis is still needed.

Conclusion

Based on the LC-ICP-MS technology, a method for analysis of four cobalamins and Co(II) in infant milk powder samples was established. The instrumental analysis for the four cobalamins and Co (II) was completed within 15 min. The samples were hydrolyzed with pepsin (pig source) and extracted into a ammonium acetate solution (pH = 4). The MDL of Co(II), OH-Cbl, CN-Cbl, Ado-Cbl and Me-Cbl ranged from 0.144 to 0.278 µg/kg with a satisfied accuracy and precision. Compared with other methods, this method is more sensitive, and pretreatment of the sample is simple and easy because it does not require solid phase extraction and follow steps. It provides a rapid, simple, and sensitive method for determination of four cobalamins and Co(II) in infant milk powder, and meets the requirement of the daily supervision of vitamin B_{12} in infant milk powder samples. The developed method provides an opportunity for researchers to make further studies about cobalamins, cobalt in infant milk powder and related matrices.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions

Jun Tang: Conceptualization, Methodology, Writing - review & editing. Xiaofeng Yu, Lijun Zhao, Chenyang Zheng and Ying Tan: Methodology, Validation. Heng Wang: Conceptualization, Writing - original draft, Writing - review & editing. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Conflict of interest

The authors have declared no conflict interest.

Additional information

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