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Determination of Cobalamin in Nutritive Supplements and Chlorella Foods by Capillary Electrophoresis−Inductively Coupled Plasma Mass Spectrometry

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A capillary electrophoresis−inductively coupled plasma mass spectrometric (CE-ICP-MS) method for the determination of cobalamin is described. Samples of cobalt-containing compounds were subjected to electrophoretic separation before injection into the microconcentric nebulizer (CEI-100) of ICP-MS. The Co-containing compounds studied include cyanocobalamin (CN-Cbl, vitamin B12), hydroxocobalamin (OH-Cbl), and Co(II). The species studied were well separated using a 70 cm length × 75 μm i.d. fused silica capillary with the applied voltage set at +20 kV. A 25 mM Tris buffer (pH 9.0) containing 15 mM sodium dodecyl sulfate (SDS) was used as the electrophoretic buffer. The CE-ICP-MS detection limits were 0.3, 0.2, and 1.7 ng of Co mL^{-1} for CN-Cbl, OH-Cbl, and Co(II), respectively. The concentrations of cobalamin compounds were determined in selected nutritive supplements and chlorella samples. A microwave-assisted extraction method was used for the extraction of these compounds. Over 92% of the total cobalt species was extracted using a 5% v/v HNO_3 solution in a focused microwave field within a period of 10 min; spike recovery was in the range of 94–105% for various species. The major cobalt species in nutritive supplements and chlorella samples was cyanocobalamin.

KEYWORDS: Cobalt speciation; cobalamin; capillary electrophoresis; inductively coupled plasma mass spectrometry; nutritive supplements; chlorella

INTRODUCTION

Cobalamin, the only vitamin that contains a transition metal, plays an important role in protein synthesis and energy metabolism. Vitamins are crucial for maintaining good health in humans; lack of sufficient amounts of any of them can cause serious diseases. To prevent cobalamin deficiency disorders, consumption of nutritive supplements and foodstuffs with cobalamin is commonly practiced. Cobalamin is present in large concentrations in some types of chlorella, which is an important source of cobalamin. In such samples determination of cobalt species is an important pursuit. The official microbiological assay methods for the analysis of water-soluble vitamins, although very sensitive, are extremely time-consuming and sometimes not completely specific (*1, 2*). Several methods of liquid chromatography (LC) and capillary electrophoresis (CE) coupled with different detection methods for cobalt speciation analysis have appeared, including UV detection (*3, 4*), electrochemical detection (*3*), flame atomic absorption spectrometry (FAAS) (*5*), fluorometry (*4, 6*), and inductively coupled plasma mass spectrometry (ICP-MS) (*4, 7–10*). The utility of capillary electrophoresis (CE) for speciation analysis is growing rapidly (*11*). CE-ICP-MS has been applied for cobalt speciation analysis in nutritive supplements (*8*). However, to our knowledge the CE-ICP-MS technique has not been reported for the speciation analysis of cobalt in foodstuffs, which is also important.

CE is especially suitable for the separation of biological macromolecules. In comparison with other chromatographic techniques, CE has several advantages such as high resolving power, small sample volume requirement, minimal buffer consumption, and high sample throughput. As a detection technique, inductively coupled plasma mass spectrometer (ICP-MS) provides the advantages of low detection limit, multielement detection, and element- and isotope-specific detection capabilities (*12, 13*). Therefore, the use of CE as a high-resolution separation technique with an ICP-MS as a sensitive element specific detector is of growing interest for analytical research. The coupling of CE with ICP-MS for trace element speciation measurements was first reported in 1995 (*14*). Interfacing of CE with ICP-MS is not straightforward; it requires a specially designed interface that should be efficient in sample introduction to the plasma without any compromise in the resolution achieved in the capillary (*15–18*). A modified microconcentric nebulizer (CEI-100, CETAC) as the sample introduction device for the CE-ICP-MS system was developed by CETAC Technologies in collaboration with Prange et al. of

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Table 1. Equipment and Operating Conditions

ICP-MS instrument	Perkin-Elmer SCIEX ELAN 6100 DRC II
plasma conditions	
RF power	1100 W
plasma gas flow	15 L min^{-1}
auxiliary gas flow	1.13 L min^{-1}
nebulizer gas flow	1.00 L min^{-1}
mass spectrometer settings	
resolution	0.7 amu at 10% peak maximum
dwell time	100 ms
sweeps/reading	10
readings/replicate	400
isotopes monitored	^{59}Co
CE-ICP-MS interface	CETAC CEI-100
liquid uptake rate	5.4 μL min^{-1}
CE system	home-made
capillary	fused silica, 75 μm i.d., 70 cm length
electrophoretic buffer	25 mM Tris, 15 mM SDS, pH 9.0
electrophoretic voltage	20 kV
sample injection	hydrodynamic method (10 cm, 60 s)

the GKSS Research Center (Germany) (*16, 17*). This commercial interface between CE and ICP-MS overcomes the degradation of species separation by nebulizer suction and provides effective analyte transport to the ICP-MS instrument and a stable electrical connection.

The aims of the present work are to develop a rapid microwave-assisted extraction procedure and an accurate method for the speciation analysis of cobalt in nutritive supplements and in chlorella samples using CE-ICP-MS. It is based on the coupling of CE with online selective detection of cobalt by ICP-MS. The eluates are directly introduced into ICP-MS for the detection of Co at *m/z* 59. The optimization of the CE-ICP-MS technique and its analytical feasibilities, and also its applications to the determination of cobalt compounds in nutritive supplements and in chlorella samples, are described.

MATERIALS AND METHODS

CE-ICP-MS System. The instrumental setup of the CE-ICP-MS system consists of an indigenously developed capillary electrophoresis unit equipped with a Spellman CZE-1000R high-voltage power supply (Spellman Electronics Corp., Plainview, NY) and an ELAN 6100 DRCII ICP-MS instrument (Perkin-Elmer SCIEX, Concord, ON, Canada). Peak areas and peak heights were measured by the Totalchrom Workstation (Perkin-Elmer). The experimental conditions of the CE-ICP-MS system are listed in **Table 1**.

A detailed description of the CEI-100 capillary electrophoresis interface (CETAC, Omaha, NE) was given in a previous paper (*17*). The nebulizer worked in the self-aspiration mode at a flow rate of around 5.4 μL min^{-1} (*13*). A 0.1% v/v HNO_3 solution was used as the makeup solution to provide the electrical connection.

CE Separation System and Conditions. A fused silica capillary (Alltech) with an inner diameter of 75 μm (70 cm length) and an outer diameter of 375 μm was used as electrophoresis capillary. The capillary was conditioned before use as described in our previous paper (*19*). Sample was injected into the CE capillary by the hydrodynamic method (*19*). The injected volume was about 8 nL for a 60 s injection. A 25 mM tris(hydroxymethyl) aminomethane (Tris) solution containing 15 mM sodium dodecyl sulfate (SDS) (pH 9.0) was used as the electrophoretic buffer, and the applied voltage was set at +20 kV.

Chemicals. Analytical-reagent grade chemicals were used without further purification. Purified water (18.2 MΩ-cm), from a Milli-Q water purification system (Millipore, Bedford, MA), was used to prepare all of the solutions. Cyanocobalamin (CN-Cbl) and hydroxocobalamin hydrochloride (OH-Cbl) were obtained from Sigma (St. Louis, MO). Inorganic cobalt [Co(II)] elemental standard solution was obtained from Fisher (Fair Lawn, NJ). Sodium dodecyl sulfate (SDS) was from J. T. Baker (Phillipsburg, NJ), and Tris, HNO_3, HCl, and NaOH were procured from Merck (Darmstadt, Germany). Stock solutions (200 μg of Co mL^{-1}) of the cobalamin species were prepared in pure water and diluted appropriately before use. The electrophoretic buffer solutions were adjusted to the desired pH using 0.1 mM NaOH or 0.1 mM HCl. All solvents and solutions for CE analysis were filtered through a PVDF filter (Millipore) of 0.22 μm porosity.

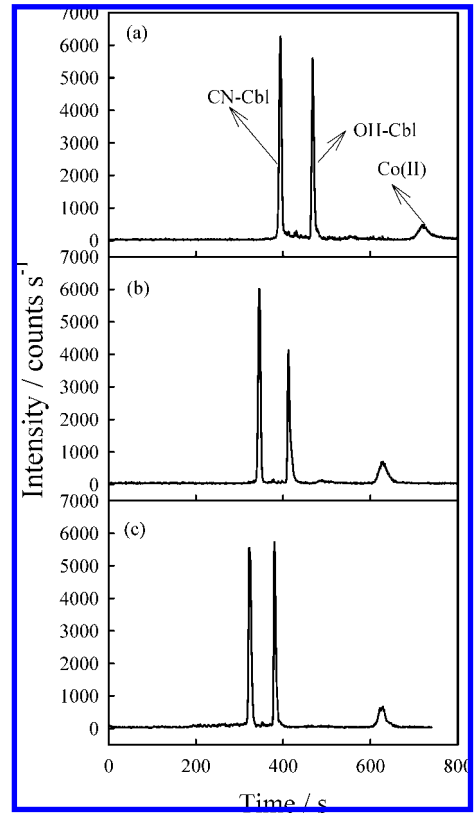

Figure 1. Effect of separating voltage on electropherogram: (**a**) 18 kV; (**b**) 20 kV; (**c**) 23 kV. Each buffer solution contained 30 mM Tris and 15 mM SDS (pH 9.0). The concentrations of CN-Cbl, OH-Cbl, and Co(II) were 50 ng of Co mL^{-1} each.

Sample Preparation and Extraction. The applicability of the method to real samples was demonstrated by the analysis of two nutritive supplements and two chlorella samples purchased locally. The sample powders were obtained by sieving to 100 mesh after they were lyophilized, homogenized, and ground. A simple and rapid microwave-assisted extraction procedure was used for the extraction of cobalamin species from the samples studied (*13*). A Star system 2 (CEM, Matthews, NC) focused microwave digester was used as the extracting device. Approximately 0.25 g of powder samples was accurately weighed into 15 mL polyethylene centrifuge tubes, and 5 mL of 5% v/v HNO_3 solution was added into each tube. The tubes were then inserted into the 250 mL calibrated round-bottom open vessel (Pyrex glass) having 80 mL of water (*13*). The microwave system was programmed to heat the water at 90 °C for 10 min, and the ramp time was set as 4 min. After microwave heating, the samples were allowed to cool and directly centrifuged for 5 min at 3500 rpm (Hettich, Germany). The supernatant of nutritive supplement 1 was diluted by another 5-fold with pure water, and all of the supernatants were filtered through a PVDF filter (Millipore) of 0.22 μm porosity before CE separation. The concentrations of various cobalt species were determined by an external calibration method based on peak area. The spike recoveries were determined by spiking 0.25 g of samples with suitable amounts of cobalamins and Co(II) standard solutions, followed by drying and extraction by HNO_3 solution. The standards spiked were 400 ng g^{-1} each in nutritive supplement 2 and chlorella 1 and 200 ng g^{-1} each in chlorella 2, whereas the spikes were 2 μg g^{-1} each of cobalamins and 20 μg g^{-1} Co(II) in nutritive supplement 1. The samples were also digested

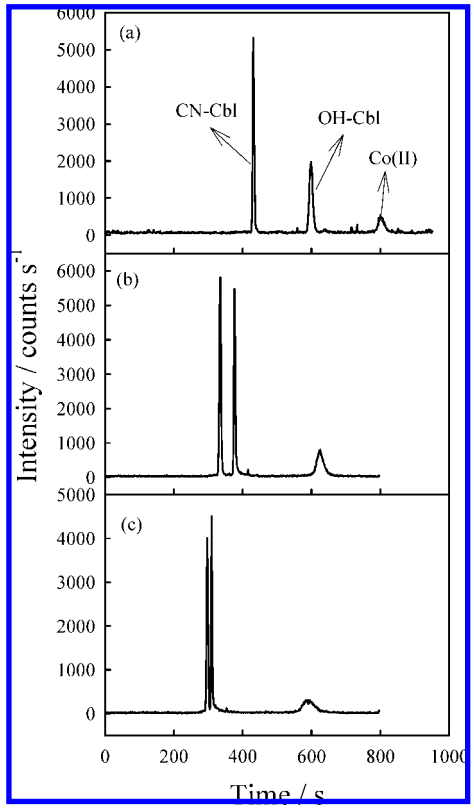

Figure 2. Effect of the pH of electrophoretic buffer on CE separation: (**a**) 8.5; (**b**) 9.0; (**c**) 9.5. Each buffer solution contained 30 mM Tris and 15 mM SDS. Separation voltage was set at 20 kV. The concentrations of CN-Cbl, OH-Cbl, and Co(II) were 50 ng of Co mL^{-1} each.

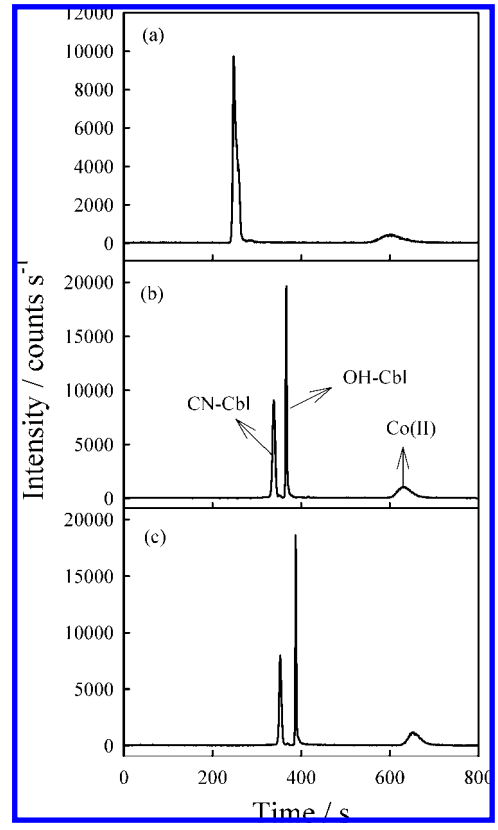

Figure 3. Effect of the concentration of SDS in Tris buffer on CE separation: (**a**) 10 mM; (**b**) 15 mM; (**c**) 20 mM. Each buffer solution was present as 30 mM Tris (pH 9.0). Separation voltage was set at 20 kV. The concentrations of CN-Cbl, OH-Cbl, and Co(II) were 50 ng of Co mL^{-1} each.

completely using pressurized microwave digestion procedure using HNO_3. The powder samples (0.5 g) were weighed into closed Teflon PFA vessels, 5 mL of concentrated nitric acid was added, and the vessels were heated inside a CEM MARS 5 microwave digester to decompose the sample. The microwave digester was operated at a power of 600 W (50%) and a pressure of 100 psi; the ramp time was 20 min, and the hold time was 10 min. After cooling, the digests were transferred into 10 mL volumetric flasks and made up to the volume with pure water. The stock solutions were diluted to the appropriate volume followed by introduction into the ICP-MS for cobalt determination. The cobalt concentration in the samples was quantified by means of external calibration with 1 ng mL^{-1} of rhodium as the internal standard. The extraction efficiency was computed by comparing the total Co determined in sample solutions prepared using microwave extraction procedure and pressurized microwave digestion procedure.

RESULTS AND DISCUSSION

Selection of CE Condition. Selections of electrophoretic buffer being one of the important parameters in CE separation, different buffers, including phosphate buffer, formate buffer, and Tris buffer with SDS used in a previous study for cobalamin separations, were tested in this work (*8*). From the experiments it was found that better resolution could be obtained when Tris buffer containing SDS was used as the electrophoretic buffer. Tris buffer containing SDS was used in the following experiments. Several parameters such as electrophoretic voltage, concentration, and pH of electrophoresis buffer and concentration of SDS in Tris buffer affected the CE separation of Co species and needed detailed study.

The effect of electrophoretic voltage on CE separation is shown in **Figure 1**. As shown, an increase of electrophoretic voltage (18–23 kV) reduced the migration time of the species studied. However, at high voltages there is a possibility of bubble formation at the interface of the separation capillary and the nebulizer that can interrupt the electrical connection. Hence, an electrophoretic voltage of 20 kV was selected as optimum. The effect of the pH of the electrophoretic buffer on CE separation was investigated in the range of pH 8.5–9.5 (8.5, 8.75, 9.0, 9.25, and 9.5). As shown in **Figure 2**, the migration time decreased with the increase of pH of buffer, which could be due to the increase of the electro-osmotic flow. To get better separation of cobalamins, pH 9 was selected. The effect of the concentration of SDS in Tris buffer on CE separation is shown in **Figure 3**. As shown, cyanocobalamin and hydroxocobalamin could not be separated when the concentration of SDS was <10 mM, possibly due to insufficient concentration of micelle available to react with CN-Cbl and OH-Cbl. CN-Cbl and OH-Cbl were well separated when 15 mM SDS was used. The sequence of cobalamin species in the CE separation was CN-Cbl and OH-Cbl, which could be due to the better interaction between micelle and the OH-Cbl. The migration times increased slightly when the concentration of SDS increased. In the following experiments 15 mM SDS was added to buffer solution. The effect of the concentration of electrophoretic buffer on CE separation was also studied using 20, 25, and 30 mM concentrations of the buffer. As shown in **Figure 4**, the migration time increased slightly with the increase of the buffer concentration, which could be due to the decrease of the electro-osmotic flow with the increase of buffer concentration. To achieve better resolution for real sample analysis, a buffer concentration of 25 mM was considered to be optimum.

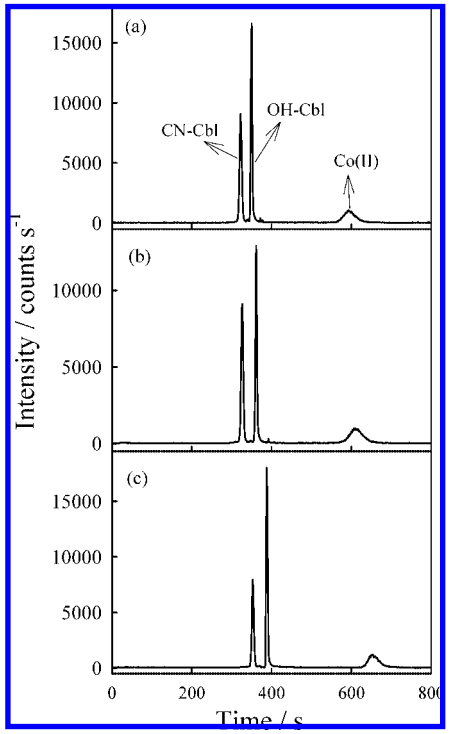

Figure 4. Effect of concentration of electrophoretic buffer on CE separation: (**a**) 20 mM; (**b**) 25 mM; (**c**) 30 mM. The concentrations of CN-Cbl, OH-Cbl, and Co(II) were 50 ng of Co mL^{-1} each. Other CE conditions are given in **Table 1**.

Table 2. Repeatability of Migration Time, Peak Area, and Peak Height of the CE Elution Peaks ($n = 5$)

species	migration time ± SD[a] (s)	repeatability of peak area[b] (%)	repeatability of peak height[b] (%)
CN-Cbl	334 ± 11	3.3	4.7
OH-Cbl	383 ± 17	4.2	5.3
Co(II)	646 ± 31	2.1	3.2

[a] Standard deviation. [b] Relative standard deviation.

A summary of the optimum operating conditions of the CE-ICP-MS system is given in **Table 1**. A typical electropherogram (ICP-MS detection) for a solution containing 50 ng of Co mL^{-1} each of three species studied is shown in **Figure 4b**. As shown, all species studied were well resolved and the separation process was completed in <11 min.

Optimized Electrophoretic Separation. At the optimized conditions, the following parameters have been computed. Repeatability was determined using five consecutive injections of a test mixture containing 20 ng of Co mL^{-1} of the cobalamin species and 200 ng mL^{-1} Co(II). As shown in **Table 2**, the relative standard deviation of the peak heights and peak areas were less than 5.3 and 4.2%, respectively, and the repeatability of migration time was better than 4.7% for all species. Peak areas estimated by calculating the areas under the elution peaks indicated that the responses for Co were similar for CN-Cbl, OH-Cbl, and Co(II). The broad Co(II) peak could be due to the strong interaction between Co(II) and the capillary wall. Calibration curves based on peak height and peak area were linear with correlation coefficient (r^2) better than 0.996 for each species in the range studied (0.002–2 μg of Co mL^{-1}). The detection limit was estimated from the peak height versus concentration plot and based on the concentration (as element) necessary to yield a net signal equal to 3 times the standard deviation of the background. The CE-ICP-MS detection limits were 0.3, 0.2, and 1.7 ng of Co mL^{-1} for CN-Cbl, OH-Cbl, and Co(II), respectively. The results were better than the previous CE-ICP-MS results (*8*) and even better than the LC-ICP-MS results (*9*).

Table 3. Spike Recoveries and Concentrations of Cobalt Species in Nutritive Supplements and Chlorella As Measured by CE-ICP-MS ($n = 3$)[a]

sample and compound	spike recovery (%)	concentration found (ng of Co g^{-1})
nutritive supplement 1		
CN-Cbl	101 ± 4	2360 ± 80
OH-Cbl	94 ± 3	ND
Co(II)	105 ± 2	ND
total Co (extraction)		2380 ± 70
total Co (mineralization)		2510 ± 50 (95%)
nutritive supplement 2		
CN-Cbl	95 ± 3	185 ± 19
OH-Cbl	97 ± 4	ND
Co(II)	94 ± 5	98 ± 12
total Co (extraction)		289 ± 13
total Co (mineralization)		310 ± 8 (93%)
chlorella 1		
CN-Cbl	98 ± 4	130 ± 5
OH-Cbl	105 ± 3	ND
Co(II)	104 ± 3	ND
total Co (extraction)		132 ± 4
total Co (mineralization)		143 ± 5 (92%)
chlorella 2		
CN-Cbl	93 ± 2	381 ± 26
OH-Cbl	96 ± 4	ND
Co(II)	101 ± 3	ND
total Co (extraction)		386 ± 12
total Co (mineralization)		418 ± 2 (92%)

[a] Values are means of three measurements ± standard deviation. [b] Not detected. [c] Determined after extraction and/or mineralization by pneumatic nebulization ICP-MS. The value shown in parentheses is the extraction efficiency.

Sample Analysis. The proposed procedure was applied to determine cobalt species in two nutritive supplements and two chlorella samples purchased from the local market. The nutritive supplements and chlorella samples were prepared by microwave-assisted extraction using 5% v/v HNO_3 solution as described under Materials and Methods. As shown in parentheses in **Table 3**, the extraction efficiencies of cobalt species were better than 94 and 92% in nutritive supplements and chlorella, respectively. The extraction efficiency was computed by comparing the total Co determined in sample solutions prepared using the microwave extraction procedure and pressurized microwave digestion procedure. The assignment of electropherogram peaks was accomplished by analyte addition experiments. Typical electropherograms of cobalt species of the extract from nutritive supplement 1 and chlorella 1 are shown in **Figures 5** and **6**, respectively. As expected, cyanocobalamin has been found to be the major species in the nutritive supplements. As shown in **Figure 6**, cyanocobalamin is the only detectable cobalt species in the chlorella extracts also. The spike recoveries from nutritive supplements and chlorella samples determined as described under Materials and Methods are given in **Table 3** As shown, recoveries were in the range of 94–105% for various cobalt species studied in different samples, indicating that no significant decomposition occurred during the extraction process. The concentrations of cobalt species in the extract were determined by external calibration method. These results are also shown in **Table 3**. The concentration of cyanocobalamin in the chlorella samples is similar to that of nutritive supplement 2, implying the suitability of chlorella as an

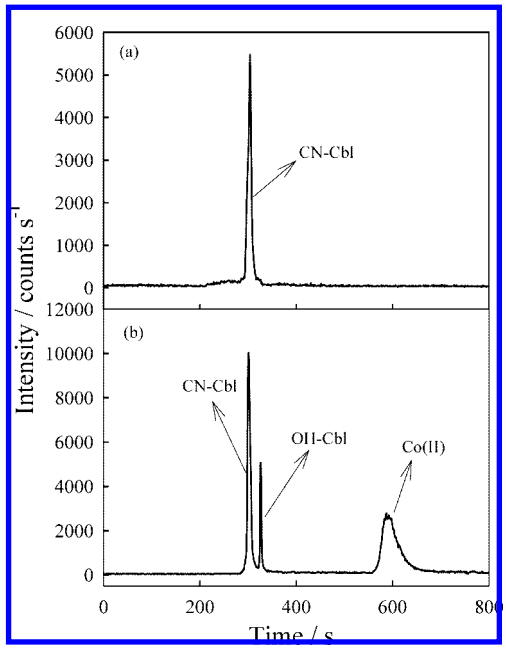

Figure 5. Typical electropherogram for the separation of cobalt species in the extract of (**a**) nutritive supplement 1 and (**b**) nutritive supplement spiked with 2 μg g^{-1} each of cobalamins and 20 μg g^{-1} Co(II).

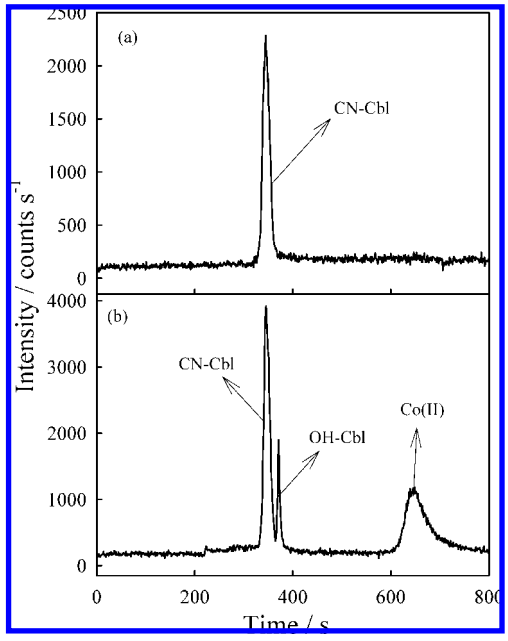

Figure 6. Typical electropherogram for the separation of cobalt species in the extract of (**a**) chlorella 1 and (**b**) chlorella spiked with 200 ng of Co g^{-1} each of cobalt species. The concentration of CN-Cbl in **a** was about 7 ng of Co mL^{-1}.

alternative nutritive supplement to vitamin B12 of natural origin. The sums of the concentrations of individual cobalt species obtained by CE-ICP-MS were in satisfactory agreement with the total cobalt concentration in the extracts and completely mineralized samples solutions (**Table 3**).

The merits of coupling CE and ICP-MS with commercially modified microconcentric nebulization for cobalt speciation in nutritive supplement and chlorella have been demonstrated. Simplicity and extraction efficiency of >92% are the promising features of the procedure. The detection limits of various cobalt species obtained with this system are low enough for the cobalt speciation analysis in real samples without complicated sample pretreatment. The system could provide a rapid and sensitive procedure for cobalt speciation analysis.

ABBREVIATIONS USED

CN-Cbl, cyanocobalamin; OH-Cbl, hydroxocobalamin; Co(II), cobalt; ICP-MS, inductively coupled plasma mass spectrometry; CE, capillary electrophoresis.

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