

This document is downloaded from DR-NTU, Nanyang Technological University Library, Singapore.

Title	Rapid determination of vitamin B12 concentration with a chemiluminescence lab on a chip
Author(s)	Lok, Khoi Seng; Siti Zubaidah Abdul Muttalib; Lee, Peter Peng Foo; Kwok, Yien Chian; Nguyen, Nam-Trung
Citation	Lok, K. S., Siti, Z. A. M., Lee, P. P. F., Kwok, Y. C., & Nguyen, N. T. (2012). Rapid Determination of Vitamin B12 Concentration with a Chemiluminescence Lab on a Chip. <i>Lab on a Chip</i> , 12(13), 2353-2361.
Date	2012
URL	http://hdl.handle.net/10220/7933
Rights	© 2012 The Royal Society of Chemistry. This is the author created version of a work that has been peer reviewed and accepted for publication by <i>Lab on a Chip</i> , The Royal Society of Chemistry. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: DOI: [http://dx.doi.org/10.1039/C2LC00037G].

Rapid determination of vitamin B₁₂ concentration with a chemiluminescence lab on a chip

Khoi Seng Lok,^a Siti Zubaidah binte Abdul Muttalib,^a Peter Peng Foo Lee,^a Yien Chian Kwok^a and Nam-Trung Nguyen^b

Received (in XXX, XXX) Xth XXXXXXXXX 200X, Accepted Xth XXXXXXXXX 200X

First published on the web Xth XXXXXXXXX 200X

DOI: 10.1039/b000000x

This paper reports a novel method for the rapid determination of vitamin B₁₂ concentration in a continuous-flow lab-on-a-chip system. This new method is based on luminol-peroxide chemiluminescence (CL) assays for the detection of cobalt(II) ions in vitamin B₁₂ molecules. The lab-on-a-chip device consisted of two passive micromixers acting as microreactors and a double spiral microchannel network serving as an optical detection region. This system could operate in two modes. In the first mode, samples are acidified and evaluated directly in the microchip. In the second mode, samples are treated externally by acidification prior to detection in the microchip. In the first mode, the linear range obtained was between 1.00 ng/ml to 10 µg/ml, R² = 0.996, with a relative standard deviation (RSD) of 1.23% to 2.31% (n = 5) and a limit of detection (lod) of 0.368 pg/ml. The minimum sample volume required and the analytical time were 30 µl and 3.6 seconds, respectively. In the second mode, the linear range obtained was between 0.10 ng/ml to 10 µg/ml, R² = 0.994, with the RSD of 0.90% to 2.32% (n = 6) and a lod of 0.576 pg/ml. The minimum sample and the analytical time required were 50 µl and 6 seconds, respectively. The lab on a chip working in mode II was successfully used for the determination of vitamin B₁₂ concentrations in nutritional supplemental tablets and hen egg yolks.

Introduction

Vitamin B₁₂ (cobalamin) is an organic complex that contains a cobalt ion in its structure.¹ This vitamin is an important coenzyme for cell development and growth, and its deficiency leads to weakness, fatigue, nausea, constipation, weight loss, pernicious anemia and nerve degeneration.² Peculiarly, vitamin B₁₂ is essential for the human diet, but cannot be synthesized by the body. This vitamin has to be obtained from natural sources such as fish, dairy products, egg, meat and poultry. However, compared to other vitamins, the daily requirement of vitamin B₁₂ is relatively low.³ The determination of vitamin B₁₂ in human serum at picogram level becomes necessary for the detection of its deficiency.⁴ Hence, sensitive analytical detection devices are required. To counter the deficiency of vitamin B₁₂, many supplements are available in the form of tablets, capsules and injections. Correspondingly, related quality checks of these products are needed. Furthermore, vitamin B₁₂ analysis is important for the determination of its quantity and activity in food and microbial fermentation broth, as well as for the assessment of chemical properties of algal vitamin B₁₂.¹

Methods such as microbiological assay,⁵ radioisotopic assay,⁶ spectrophotometry,⁷ high-performance liquid chromatography,⁸⁻¹⁰ fluorimetric assay,¹¹ capillary electrophoresis,^{12, 13} chemiluminescence (CL),¹⁴⁻¹⁶ enzyme-linked immunosorbent assay¹⁷ and surface plasmon resonance-based biosensor assay¹⁸ were developed for quantifying Vitamin B₁₂ for various applications. Among all these different methods, the sensitivity of the CL-based assay is the highest¹ and can be as low as 5 pg/ml.¹⁴ Qin *et al.* first introduced this concept in 1997. The injected vitamin B₁₂ sample was acidified with diluted HCl in a mixing coil, followed by pH adjustment with Na₂CO₃. Next, the acidified vitamin B₁₂ sample was mixed with luminol and ad-hoc generated H₂O₂. Finally CL signal was presented to the flow cell sensor.¹⁹ Sou and Hou reported a similar setup, but they enhanced the CL reaction with dissolved oxygen and performed pre-treatments to the samples.²⁰ Akbay and Gok introduced a new external

acidification method followed by the regular FIA-CL sensor.¹⁶ Recently, Kumar *et al.* reported a CL-based method using urea-H₂O₂ for determining the concentration of vitamin B₁₂ using a luminometer platform.¹⁴ All these works aimed to advance the chemical procedures associated with the conventional FIA-CL sensors or luminometers. Despite the numerous vitamin B₁₂ assays that are currently available, there is a need for improved assays to overcome the shortcomings of the above state-of-the-art techniques.¹ These shortcomings include the lack of sensitivity, difficulties encountered in standardization of test procedure, and the impracticable use of high-volumes in certain situations (e.g., in hospitals). Moreover, commercial non-isotopic immunoassays for the detection of vitamin B₁₂ are not available. The recent advancement of lab-on-a-chip technology would allow the implementation of a vitamin B₁₂ assay into microfluidic system.

Lab-on-a-chip (LOC) or micro total analysis system (µTAS) aims to implement chemical analysis onto a miniature platform. Microchannel networks can be integrated into a microchip to allow mixing of reagents, reaction and detection. Specialised microchips had been made for CL detection in an array of analytes, including the determination of hydrogen peroxide in rainwater,²¹ glucose,²² luciferase,²³ cobalt(II) ions,²⁴ benzyl peroxide in flour²⁵ etc. Microfluidic devices offer rapid analysis with high sensitivities, low costs and parallelization, which can address the shortcomings of the existing vitamin B₁₂ assays.

CL-based assays, utilizing luminol, lophine or gallic acid can be used for the detection of cobalt ions. Cobalt(II) ion serves as an effective catalyst in the oxidation of luminol, in the presence of hydrogen peroxide in alkaline medium condition. This reaction emits blue light at a wavelength of 425 nm. But, the cobalt in vitamin B₁₂ complex itself does not catalyse the CL reaction. The solution of vitamin B₁₂ has to be acidified to set the cobalt ions free for catalysis and detect presented ions. The amount of cobalt(II) ions detected correlates directly to the concentration of the vitamin.¹⁵ Therefore, the acidification process is crucial for the determination of the vitamin presented. A variety of methods have been implemented. Kumar *et al.*

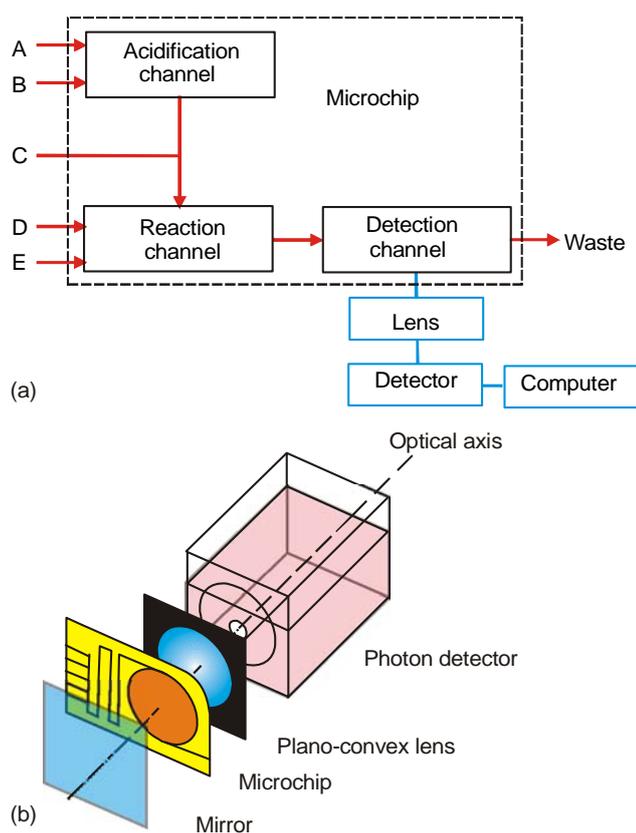


Fig. 1 Detection concept and experimental setup: (a) Schematic diagram of the flow system; (b) The setup with the lens holder, the mirror, the microfluidic device and the photon detector. **The total device dimension is 50 mm×50 mm×100 mm.**

acidified the vitamin B₁₂ solution with 9 M of HNO₃. Excess HNO₃ was then removed with 5.5 M of HCl, followed by dilution with bicarbonate buffer (pH 10).¹⁴ Akbay and Gok used an equal ratio of HNO₃, and HCl neutralized with 0.2 M of NaOH.¹⁶ Qin *et al.* used only 0.2 M of HCl without heating to dryness. The samples were then neutralized directly with 0.6 M of sodium carbonate in the mixing coil.¹⁹ Song and Hou used 0.1 M HCl to acidify crystalline vitamin B₁₂, its pharmaceutical products and human serum externally.²⁰ The use of harsh corrosive acids may be better for liberating the cobalt ions from the vitamins in the samples, and hence may give better dynamic ranges and lower detection limits. However, harsh reaction conditions inevitably harbour high occupational hazard risks and may not be essential or necessary to achieve the purpose of vitamin B₁₂ determination.

In this paper, we first explored the use of a continuous-flow microfluidic device for the CL detection of cobalt(II) ions in vitamin B₁₂. A LOC device containing two passive micromixers and a double spiral microchannel network was designed and fabricated. The micromixer resolved the mixing issue of the laminar flow of a fluid in microchannels, allowing rapid reagents and sample mixing and faster detection.²⁴ The double spiral microchannel network allows a better presentation of CL signals to a photon detector as compared to a single spiral in the conventional flow cell. This device was specially designed to embrace two current laboratory practices. In the first practice, the vitamin B₁₂ sample was mixed directly with diluted HCl in an

acidification channel, followed by its immediate detection using a luminol-peroxide CL assay in the microchannel network. In the second practice, the vitamin B₁₂ samples were externally acidified in a digester. The acidified extracts were then quantitatively analysed using the CL assay in a microfluidic system. A milder acidification procedure was also introduced and tested. Real samples using vitamin B₁₂ tablets and hen eggs were analysed. The LOC device shows good correlations between the emitted CL intensities and vitamin B₁₂ concentrations. The device reported here is small in size, light in weight, portable and has an analysis performance comparable to current FIA/CL detection instruments. These features allow the use of our system in the field and at remote locations.

Experimental

Conceptual design of the microfluidic system

Figure 1(a) shows the schematic diagram of the detection concept. The continuous-flow system consists of programmable infusion pumps, a microchip, and a detector. The infusion pumps introduce the reagents continuously. The microchip houses the microchannel network. The detector records a steady CL signal emitted from the reaction. The conventional luminol-peroxide CL chemistry was adopted. The system was designed to operate in two modes, I and II. In mode I, vitamin B₁₂ sample was directly acidified in the microfluidic device. Vitamin B₁₂ sample, hydrochloric acid (HCl), sodium hydroxide (NaOH), luminol and hydrogen peroxide (H₂O₂) were introduced into the system through inlets A, B, C, D and E respectively, Fig. 1(a). Vitamin B₁₂ sample was mixed with diluted HCl to liberate the cobalt(II) ions in the acidification channel. Next, NaOH was introduced to neutralize the acidified sample. This mixture was then mixed with luminol and H₂O₂ in the reaction channel. The emitted CL signal from the mixture was detected in the detection channel. In mode II, the acidification of the vitamin B₁₂ sample was carried out outside the microfluidic system. The inlets A and B of the microchip were blocked. The treated vitamin B₁₂ sample, luminol and hydrogen peroxide (H₂O₂) were introduced into the system through inlets C, D and E respectively, Fig. 1(c). The vitamin B₁₂ sample was mixed with luminol and H₂O₂ in the reaction channel.

A multi-syringe (KDS220, KD Scientific, MA, USA) and a double-syringe infusion pump (KDS200, KD Scientific, MA, USA) was used to deliver the reagents such as HCl, NaOH, luminol and H₂O₂. Another single-syringe infusion pump (KDS100, KD Scientific, MA, USA) was used to deliver the test sample, in this case, the acidified vitamin B₁₂ solution, deionised water or hydrochloric acid (HCl). The reagents were stored in disposable 5-ml Terumo syringes to reduce cross-contaminations. Although, the different inlet streams may be adjusted to run at different flow rates, the same flow rate was used in our work to simplify the operation of the system. The flow rate of each syringe was set at 0.5 ml/min, giving a total flow rate of 2.5 ml/min and 1.5 ml/min in the mode I and II, respectively.

A lens holder (50 mm×50 mm×100 mm) was made to house the backing mirror, the microchip, the plano-convex lens and the photon detector (H7467 with microcontroller and RS-232C interface, Hamamatsu, Japan), Fig. 1(b). The CL light emitted from the reaction was scattered in all directions and dimmer as compared to the environment light. Hence, it is important to direct most of the light back to the detector to maximize the sensitivity, and concurrently shielded the detector from any background light. The backing mirror reduced the background

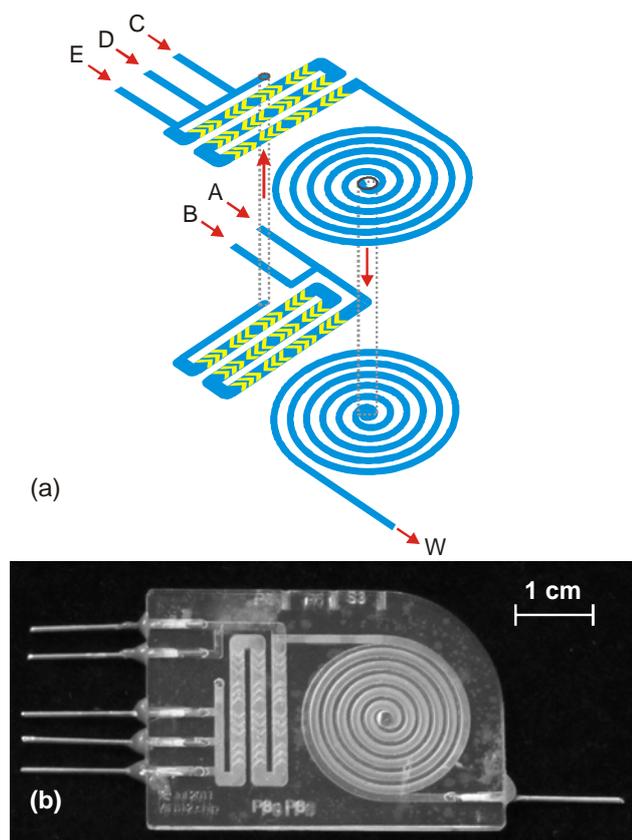


Fig. 2 Lab on a chip for determination of vitamin B₁₂ concentration: (a) microchannel network (A to E are inlets; W is outlet to waste; arrows indicate the fluid flow); (b) Fabricated microchip. The chip measures 45 mm×30 mm×3 mm.

and reflected the CL light back to the detector. A plano-convex lens (30 mm in diameter, 30 mm focal length, VIS0 coating, Edmund optics, Singapore) focuses the scattered CL light into the 8-mm aperture of the photon detector. The detector is based on a photomultiplier tube (PMT) sensor, made especially for low-level light detection. The sensor is sensitive for wavelengths between 300 nm and 650 nm, highly responsive to typical 420 nm light at 270,000 per s.p.w. Integration time can be set between 10 to 10,000 ms.

Figure 2(a) depicts the microchannel network of the microchip. This network was fabricated into three layers. The first layer works as the cover for the microchannels in the second layer. The microchannel network in the second layer consists of a passive micromixer and a clock-wise spiral microchannel for detection. The third layer contains another passive micromixer together with a matching anti-clock-wise spiral microchannel. The passive serpentine micromixer is 62 mm long, 1.8 mm wide and 0.4 mm deep. The herring-bone structure has a height of 0.2 mm and a width of 0.5 mm. This micromixer adopts the working principle of chaotic advection similar to our previously published work.²⁴ The micromixer has two periods of herring-bone structure in each turn, providing six periods of mixing modules. The gap between the parallel channels is 1 mm, and the turning width of the channel is 2.8 mm. The double spiral was formed by a clock-wise spiral and an anti-clock-wise spiral which overlaid each other perfectly. The spiral channels are connected by a via hole in the centre of the spirals. The spiral channels have a height of 0.9 mm and a width of 1 mm. The spiral channels

form a circular area with a diameter of 22 mm and an effective volume of approximately 150 μ l for CL detection.

Micro fabrication

The LOC device and the holder were designed using CorelDraw (Corel Co., Canada), a vector graphics software. A commercial CO₂ laser system (Universal M-300, Universal Laser Systems Inc., Arizona, USA) was used to transfer the designs to a transparent Polymethyl methacrylate (PMMA) substrate. For the lens holder, a 2-mm thick PMMA sheet was used. The machined parts were assembled and covered with black paper. PMMA sheet of 1-mm thickness was used for the LOC device. After machining, the parts were washed in 99% denatured ethanol for 30 seconds, followed by rising in running deionised water for a minute and wiped cleanly. A high pressure air gun was used to remove any remaining dust particles. The PMMA parts were subsequently aligned and assembled. The parts were then secured with masking tapes. The assembled parts were subsequently sandwiched between two clean polished silicon wafer and thermally bonded at 165°C and low pressure.²⁶ Metal tubes were attached to the device by epoxy adhesive to work as inlets and outlets. The final dimensions of the fabricated microchip were 45 mm in length, 30 mm in wide and 3 mm in thickness.

Data analysis

The minimum sample volumes required for the analysis in modes I and II were 30 μ l and 50 μ l, respectively. The corresponding minimum analytical times were 3.6 s and 6 s for modes I and II, respectively. In order to achieve steady CL signals longer analysis duration was used in our experiments. The CL signals were captured by the photon detector at a 50-ms time intervals. This time interval was chosen to detect the signal produced by the investigated concentrations of the analyte without saturation. A customized program was written to log the captured data points in a computer. A sample size of at least 1000 data points was collected for each run, and each experiment was repeated at least five times. The data were subsequently imported and analyzed using Microsoft Excel. The photon counts were averaged and standard deviations were calculated. All the experiments and readings were conducted and recorded in a dark room to reduce background noise. Background signals were recorded by running the CL assay in the system with deionized water. A solution of 1.0 M HCl was used to flush out the residual cobalt(II) ions in the microchip between each experimental run.

Chemical reagents

All the reagents were of analytical grade, and prepared fresh before each experiment. Ultrapure water (18.2 m Ω /cm) was obtained from a Milli-Q purification system (Millipore, Japan). Luminol and pure crystalline vitamin B₁₂ were purchased from Sigma-Aldrich (USA). 30% v/v hydrogen peroxide (H₂O₂) and bicarbonate buffer (pH 10) were obtained from Scharlau (Spain). A stock solution of luminol (10 mM) was prepared by dissolving the luminol in 1.5 ml of 0.1 M sodium hydroxide made up to 10 ml in bicarbonate buffer. Further dilutions of this stock solution were prepared using bicarbonate buffer to obtain concentrations of 1 μ M to 1 mM luminol solutions for the analysis. An amount of 1 mg of vitamin B₁₂ was dissolved in 1 ml of bicarbonate

buffer to give a stock solution of vitamin B₁₂ at 1 mg/ml. This stock solution was either acidified or used directly. Further dilutions of this stock solution were prepared in bicarbonate buffer to obtain concentrations of 1 pg/ml to 1 µg/ml of vitamin B₁₂ after the acidification.

Acidification of vitamin B₁₂

In mode I, using the same working principle in Qin *et al.*'s work,¹⁹ 0.2 M HCl was used to acidify vitamin B₁₂ directly in the microchip. In mode II, a milder acidification method was devised from a combination of the work done by Kumar *et al.*¹⁴ and Akbay and Gok.¹⁶ 0.4 ml of the stock solution (1mg/ml) was added to 4 ml of 5M nitric acid (HNO₃) and heated to dryness on a heating magnetic stirrer inside a fume hood. Subsequently, 4 ml of 3M HCl was added to remove any traces of HNO₃ in the solution, giving a concentration of 100 µg/ml of acidified vitamin B₁₂. The solution was then heated at approximately 95°C for 4 minutes and cooled. The actual concentration of the acidified stock vitamin B₁₂ was verified by UV-visible spectrophotometry. The residue was subsequently diluted to concentrations of 1 pg/ml to 1 µg/ml in bicarbonate buffer.

Preparations of vitamin B₁₂ supplements and egg yolk

The preparation process of the samples were adopted from Hou and Song.²⁰ Vitamin B₁₂ tablets were obtained from a local nutraceutical retailer, GNC. The tablets were weighed and grounded to a fine powder. An amount of 500µg of vitamin B₁₂ powder was accurately weighed and dissolved into 100 ml deionised (DI) water in a clean, sterilised conical flask. An amount of 0.5 ml of this prepared vitamin B₁₂ solution was then added to 5.0 ml of HCl and further diluted with 25 ml DI-water in a 25-ml flask. Eggs were purchased from a local supermarket. They were pre-treated according to the literature.²⁷ Approximately 5.0 g of boiled egg yolk was weighed, grounded and acidified with 50 ml of 0.5 M HCl acid in a digester. The egg yolk was then digested ultrasonically until a homogeneous mixture was obtained. The mixture was centrifuged and the supernatant was obtained and filtered. The filtrates were then further analysed. All processed samples were analyzed by our CL lab-on-a-chip system and UV-visible spectrophotometry. With the determination of vitamin B₁₂ in UV-visible spectrophotometry, a calibration curve was obtained by serial dilutions of the vitamin B₁₂ solution at a wavelength of 361 nm.²⁸ The concentrations of vitamin B₁₂ in the samples were determined by comparing the recorded value to this calibration curve.

Results and discussion

Fabricated microchip

As mentioned above, the LOC devices were fabricated in polymethyl methacrylate (PMMA) substrate, using laser ablation and thermal bonding, Fig. 2(b). PMMA is a well suited material to use for this application as compared to other polymeric substrates.²⁹ The inert and hydrophobic surface of PMMA leads to minimum interaction with the reagents. Therefore, this material provides an excellent resistance against alkaline and acidic chemicals. This property is especially important for a vitamin B₁₂

assay as the samples are treated in an acidic medium, followed by the detection in an alkaline medium. A 3-mm thick PMMA sheet has a transmission of visible light up to 92% leading to a good sensitivity for CL measurement.

Optimization of reaction conditions

Since the microchip was designed to operate in two different modes, the device has to be optimized separately. In mode I, the system has five inlets (Fig. 2(a)) which determine the flow rates of the reagents and need to be optimized accordingly. The flow rates of the reagents affect the final pH level of the mixture. In mode II, the system has three inlets. The concentration of H₂O₂ and luminol are the main factors that affect the CL reaction.

Effect of flow rate in mode I. First, the optimum flow rate of the microfluidic system was determined. Reagents of 0.2 M HCl, 1 µg/ml vitamin B₁₂, 0.2 M NaOH, 1 mM Luminol and 3% v/v H₂O₂ were introduced into the microchip at flow rates ranging from 0.5 to 3.5 ml/min. The CL intensity recorded against the different flow rates are shown in Fig. 3(a). As expected, CL intensity increased with increasing flow rates from 0.5 to 2.5 ml/min. However, if the flow rate increases beyond 2.5 ml/min, the CL intensity decreases drastically from 3.2×10⁴ to 3.7×10³. At a high flow rate, the residence time in the micromixer becomes shorter (1.037 seconds for a flow rate of 2.5 ml/min), and the acidified samples may not mix well with NaOH before entering the detection channel. The subsequent luminol CL reaction was carried out in an unfavourable acidic condition³⁰ resulting in a poor CL signal. Since the flow rate of 2.5 ml/min gave the highest CL signal, this flow rate was used in our subsequent experiments.

Effect of HCl concentration in mode I. The concentration of HCl played an important role in mode I. To investigate the effect of HCl concentration, solutions of 0 to 0.5 M HCl, 1 µg/ml vitamin B₁₂, 0.2 M NaOH, 1 mM luminol and 3% v/v H₂O₂ were introduced into the system. Figure 3(b) shows the effect of HCl on the CL intensity. CL intensity increased from 0 M to 0.2 M HCl, because the amount of liberated cobalt ions increases with more a higher acid concentration. However, the CL intensity decreased rapidly from 0.2 M to 0.5 M HCl, because the excess acid alters the pH condition unfavourable for the subsequent luminol-CL reaction.

Effect of NaOH concentration in mode I. The concentration of HCl was fixed at 0.2 M to study the effect of NaOH concentration on this system. NaOH can neutralise the acidified sample. Furthermore, it can be used to adjust the pH level of the reaction. Solutions of 0.2 M HCl, 1 µg/ml vitamin B₁₂, 0 to 1 M of NaOH, 1mM Luminol and 3% v/v H₂O₂ were introduced into the system. The effect of NaOH on CL intensity is shown in Fig. 3(c). Figure 3(c) clearly shows that 0.2 M of NaOH gave the highest CL signal. At low concentrations of less than 0.2 M NaOH, the amount of NaOH introduced may not be sufficient to neutralize the acidified sample completely. At high concentrations of more than 0.2 M NaOH, the amount of NaOH makes the pH level too alkaline, which is unfavourable for CL reaction.

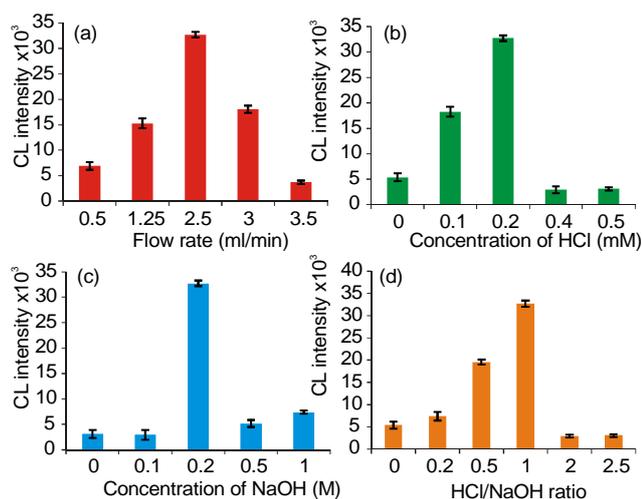


Fig. 3 Optimization of reaction condition in mode I: (a) CL intensity versus flow rate; (b) CL intensity versus concentration of HCl (at a flow rate of 2.5 ml/min for all reagents); (c) CL intensity versus concentration of NaOH (at a flow rate of 2.5 ml/min for all reagents and HCl concentration of 0.2 M), (d) CL intensity versus molar ratio of HCl to NaOH.

Further investigation was done on how the addition of acid might have changed the pH level in a carbonate buffer. Experiments were carried out using test tubes and a pH meter. The solution of 0.1 M HCl was added to carbonate buffer in different portions. Table 1 shows how the pH of the solution that may be affected by different amounts of HCl. The result explains why the CL reaction was suppressed when NaOH failed to mix well and to neutralize the acidified sample at high flow rates, Fig. 3(a). A high concentration of HCl also suppresses the CL reaction, because NaOH cannot neutralize the acidified sample completely, Fig. 3(b).

Ratio of HCl to carbonate buffer	pH
1:1	1.85
1:2	6.02
1:3	6.81
1:4	7.33
0:1	9.94

The molar ratio between HCl and NaOH can significantly affect the CL intensity, Fig. 3(d). If the ratio of HCl to NaOH increases from 0 to 1, the CL intensity also increases. This phenomenon can be explained the fact that the cobalt(II) ions were better liberated with a stronger acid. When the equal amount of HCl and NaOH was used, the CL intensity was the highest, because a mole of HCl is required to neutralize a mole of NaOH to give a neutral salt at pH 7. Hence, the amount of NaOH added to HCl must be in equal or more, to achieve a pH level of 7 or higher. Surprisingly, additional amounts of NaOH did not seem to aid the reaction, because a high pH level inhibits the CL reaction.³⁰

Effect of flow rate in mode II. The flow rate in mode II was determined separately because in this mode three reagents were used instead of five. Various flow rates from 0.5 to 3.5 ml/min were used to introduce 0.1 µg/ml vitamin B₁₂, 1 mM luminol and 3% H₂O₂ into the system. The recorded CL intensity increased with an increasing flow rate used, giving a similar result as our previous finding.²⁴ This is simply because more reagents were pumped into the system per unit time leading to a brighter CL

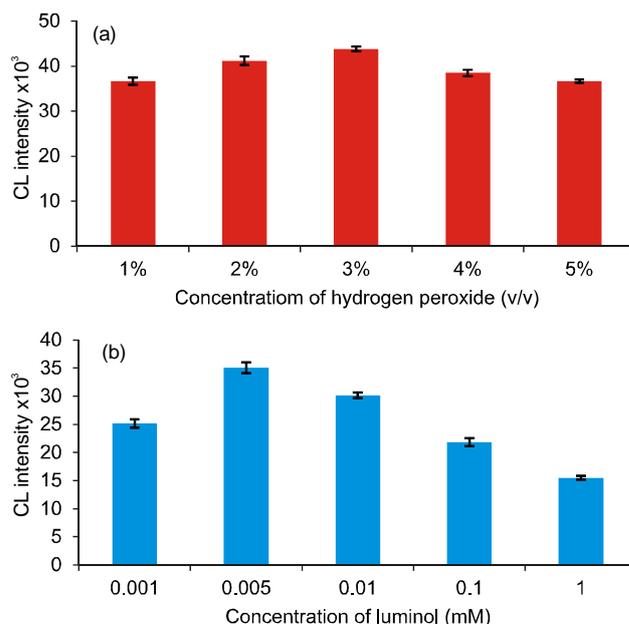


Fig. 4 Effect of concentration of (a) H₂O₂ and (b) luminol on CL intensity in mode II.

intensity. The best flow rate was determined to be 1.5 ml/min to keep a minimum analytical time of approximately 6 seconds. This flow rate was used for all the subsequent experiments related to the usage of mode II. A higher flow rate may be set for a shorter analytical time, but considering that the treated sample can be more viscous than an aqueous solution the infusion pump can stall when introducing the samples.

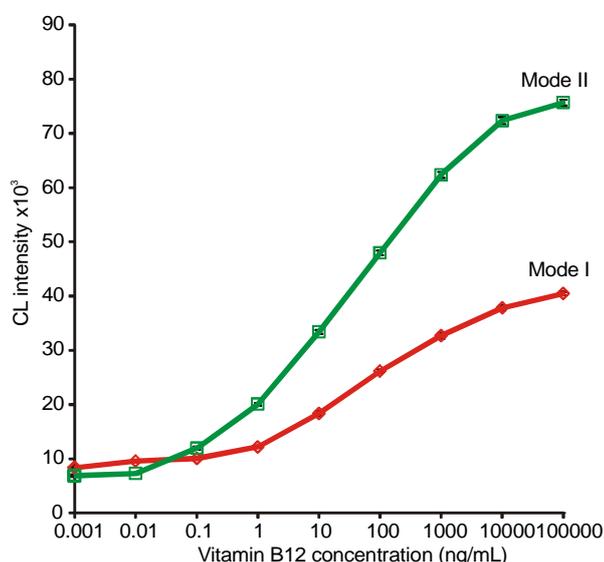
Effect of hydrogen peroxide in mode II. Next, the effect of the concentration of H₂O₂ from 1% to 5% v/v on CL intensity was investigated. Solutions of 5 µM luminol and 0.1 µg/ml vitamin B₁₂ were introduced into the device. Figure 4(a) illustrates the effect of the concentration of H₂O₂ on CL intensity emitted from the reaction. Since the CL intensity peaked at 3% v/v H₂O₂, this concentration was used in subsequent experiments.

Effect of luminol in mode II. Finally, the concentration of luminol in mode II was optimized. Various concentrations of luminol from 1 µM to 1 mM, together with 3% v/v H₂O₂, and 0.1 mg/l vitamin B₁₂ were introduced into the microfluidic system. Figure 4(b) shows the CL intensity emitted versus the concentration of luminol. The CL intensity increased with increasing concentrations of luminol, reaching a maximum at 5 µM, followed by a decline as the concentration of luminol increased further. Since the CL intensity reached a maximum at 5 µM of luminol, this concentration was used in the later experiments.

Effect of vitamin B₁₂ concentrations in mode I and II. To investigate if vitamin B₁₂ can be determined using the microfluidic system by chemiluminescence, a range of concentrations of vitamin B₁₂ samples from 1 pg/ml to 0.1 mg/ml was introduced into the system. In mode I, the vitamin B₁₂ samples were directly acidified in the microchip. The samples, 0.2 M HCl, 0.2 M NaOH, 1 mM luminol and 3% v/v H₂O₂ were introduced into the microfluidic system at a flow rate of 2.5 ml/min. The HCl solution acidifies the vitamin B₁₂ samples, liberating the cobalt(II) ions *in situ*. The liberated ions will in turn

Table 2 Vitamin B₁₂ presents in samples

Samples	Concentration of vitamin B ₁₂			
	Label	Literature	Proposed CL method	UV-visible spectrophotometry
Vitamin B ₁₂ tablets (<i>n</i> = 5)	6.40 × 10 ⁻⁸ M	—	6.20 ± 0.23 × 10 ⁻⁸ M	6.30 ± 0.35 × 10 ⁻⁸ M
Hen egg yolks (<i>n</i> = 3)	—	3 ~ 10 × 10 ⁻⁸ g/g ³¹	9.70 ± 1.10 × 10 ⁻⁸ g/g	9.90 ± 1.30 × 10 ⁻⁸ g/g

**Fig. 5** Effect of vitamin B₁₂ concentration on CL intensity

catalyse the CL reaction of luminol and peroxide in carbonate buffer (pH 10). NaOH was added to adjust the pH condition so that the CL reaction might be carried out in an alkaline medium. The emitted CL signal was captured by the photon detector and analysed. In mode II, the vitamin B₁₂ solutions were first acidified to release the initially bound cobalt(II) ions. The acidified samples, 3% v/v hydrogen peroxide and 5 μM luminol were delivered into the microfluidic system at a flow rate of 1.5 ml/min. The CL reaction was conducted in pH 10 carbonate buffer. Figure 5 shows the effect of vitamin B₁₂ concentrations on CL intensity for both modes.

For mode I and II, CL intensity increased with increasing amounts of vitamin B₁₂. For mode I, a linear range between 1 ng/ml and 10 μg/ml was observed from the curve (Fig. 5) with a relationship of $y = 6.56 \times 10^3 \cdot \lg(x) + 1.23 \times 10^4$, $R^2 = 0.996$ where *y* and *x* represented CL intensity in counts and concentration of vitamin B₁₂ in ng/ml respectively. The relative standard deviation (RSD) was between 1.23 % and 2.31% (*n* = 5). Similarly, for mode II, a linear range was observed between 0.1 ng/ml and 10 μg/ml from the curve, Fig. 5. The first order correlation equation obtained was $y = 1.27 \times 10^4 \cdot \lg(x) + 2.23 \times 10^4$, $R^2 = 0.994$. The RSD was between 0.898% and 2.32% (*n* = 6). Comparing the gradients of the two lines obtained from the two modes, the gradient of mode II is 1.93 times more than that of mode I. Therefore, mode II is more sensitive than mode I. That means acidifying the vitamin B₁₂ externally actually produces better results. However, mode I offers a more rapid and direct analysis, when the aqueous samples of vitamin B₁₂ are used.

The background signal was measured using deionised water in replacement of the vitamin B₁₂ sample. The background signal measured was 4,402 ± 488 and 4,426 ± 471 counts for mode I and II respectively. The similar values indicate that the contributions of

the background noise to the data are constant. The limit of detection (lod) was determined by the concentration of analyte required to give a signal equal to the background (blank) plus three times the standard deviation of the blank. For a concentration of 1 pg/ml of vitamin B₁₂, the detected signal was 8,370 ± 150. Using first order fitting, the lod was determined for a signal of 5,866 to be 0.368 pg/ml for mode I. Similarly, for mode II, the lod was estimated to be 0.576 pg/ml. If 1 M HCl was used instead of water, the detected signal was 2,100 ± 164 for mode I, and 2,740 ± 104 for mode II, respectively. Normally, the detection limit is lower and close to the lower value of the dynamic linear ranges, Table 3. However, this is not the case for our experiments. The photon detector used in our experiments was not able to pick up slight variations in the signal at sub-picogram level, because of the set integration over time. The integral time may then be extended to capture more photons, with better isolation from the strayed background light. However, high concentrations of analytes may also overexpose the sensor. A dynamic routine can be written to automatically adjust the integration time with the detected signal. A better photon multiple tube (PMT) with a typical sensitivity of a PMT is 4~5 × 10⁵ per s.p.W, which is 1.6 times more than the current detector. However, a more sensitive PMT will increase the cost of the device.

Determination of vitamin B₁₂ in nutritional supplements and egg yolk

Since mode II gave better detection capabilities, the proposed method was used to further determine the concentrations of vitamin B₁₂ present in real samples of vitamin B₁₂ supplemental tablets and in hen egg yolks. The preparation of these samples was discussed earlier in experimental section. UV-VIS spectrophotometry was used as a reference method for comparison.

Table 2 shows the vitamin B₁₂ present in samples of vitamin B₁₂ supplemental tablets and in hen egg yolks obtained, using the proposed CL and the reference methods. In both supplemental tablets and egg yolks, the results obtained from the proposed CL methods deviated slightly from the value indicated on the nutritional label, reference value obtained from the literature³¹ and data obtained from the UV-VIS spectrophotometry. These differences were found to be statistically insignificant ($P > 0.05$) when the two sample groups were made to undergo two-tailed unpaired T-tests. The CL microfluidic system showed promising analytical results for the determination of vitamin B₁₂ concentrations in both supplemental tablets and egg yolks.

Further experiments can be conducted to study the feasibility of this method in the quantification of vitamin B₁₂ in human serum and other naturally occurring food sources. This method may then be applied to the healthcare industry or in the quality control of food products.

Acidification of vitamin B₁₂. The acidification of vitamin B₁₂ is crucial in order to liberate the cobalt(II) ions from its organic complex. The freed cobalt(II) ions can then take part in CL-based assay. Prior to this, pre-treatment of samples were required to

Table 3 List of CL-based detection method for determination of vitamin B₁₂

Authors	Year	CL Method	Ranges (mg/ml)	lod (mg/ml)	Min. analytical time (sec)
Qin <i>et al.</i> ¹⁹	1997	Flow sensors	$1.00 \times 10^{-6} \sim 1.00 \times 10^{-2}$	3.50×10^{-7}	60
Song and Hou ²⁰	2003	Flow injection	$2.00 \times 10^{-10} \sim 1.20 \times 10^{-6}$	5.00×10^{-11}	30
Akbay and Gok ¹⁶	2008	Flow injection	$8.69 \times 10^{-6} \sim 8.69 \times 10^{-5}$	8.69×10^{-7}	10
Kumar <i>et al.</i> ¹⁴	2009	Luminometer	$1.00 \times 10^{-8} \sim 1.00 \times 10^{-2}$	5.00×10^{-7}	60
<i>Our current method</i>	2011	LOC-Mode I	$1.00 \times 10^{-6} \sim 1.00 \times 10^{-2}$	3.68×10^{-10}	3.6
		LOC-Mode II	$1.00 \times 10^{-7} \sim 1.00 \times 10^{-2}$	5.76×10^{-10}	6

extract the vitamin B₁₂ from binding proteins through heating, enzymatic digestion or ethanol precipitation from solid animal products, food samples, human plasma, bile, and feces.¹ In these treatments, the pH level was adjusted to an acidic condition of 4.8, thus allowing the release of the cobalt(II) ions indirectly. In this case, module II of the microfluidic system can be useful for such samples.

The effectiveness of the liberation of cobalt(II) ions is highly dependent on the concentrations of HNO₃ and HCl. The method used in mode II was devised from a combination of Kumar *et al.*¹⁴ and, Akbay and Gok.¹⁶ The key difference of our current method compared to the previous works are the smaller amount of corrosive acids and thus safer in handling.

Limits and merits of our system. The main objective of this study was to develop a LOC system for the determination of vitamin B₁₂ concentration. A common well-known reaction, luminol-peroxide CL, was adopted in our study. It would have been too ambitious and redundant to explore new chemical procedures and developing the LOC system simultaneously. We are confident that the working chemical procedures reported by other^{14, 16, 19, 20} to be able to function in our system and perhaps give even better results than the current procedure. Sample determination in pharmaceuticals, human serum, egg yolk and fish tissue will be feasible as well. Our present work is vastly different from the previously published works. The main concept of Qin *et al.*¹⁹ aimed at a reusable system. The experiments were done in a connected anion-exchange and glass flow cell columns. The column with immobilized luminol on resins could be used over 500 times with ad-hoc generated peroxide. There is only one mode that is using HCl to mix with vitamin B₁₂ injection samples. In our current work, we pioneered the initiatives to fabricate the traditional CL assays into a continuous-flow LOC system for vitamin B₁₂ determination. Moreover, we also introduced a new concept of designing a dual-mode chip for two laboratory practices. Most chip designs are now specialised only for a specific assay, which in turn limits the usage of the chip. Compared to our previous work,²⁴ the length of each micromixer was shortened from 202 mm to 62 mm. This design reduced the size of the microchip without hindering its performance.

Although inference study of the CL assay was not done in our work, we could easily postulate the results based on the current literatures.^{14, 19, 20} We would expect equal amount of Co²⁺, Cu²⁺ and Fe³⁺ to interfere heavily with the determination of vitamin B₁₂. These cations are well-known metallic catalysts for the luminol CL reaction. The tolerable concentration ratios with respect to 10 ng/ml vitamin B₁₂ for interference at the 5% level would be more than 1000 for ions such as Al³⁺, Ag⁺, Ba²⁺, Ca²⁺, Cd²⁺, Cr³⁺, Fe²⁺, Hg²⁺, Mg²⁺, Mn²⁺, Ni²⁺, Pb²⁺, Sn²⁺, Sr²⁺ and Zn²⁺. These unbounded ions will mildly interfere with the assays. However, they are not typically found in human serum. High concentrations (with tolerable ratio of over 1.0×10^6) of Ac⁻, Cl⁻, F⁻, HCO₃⁻, NO₃⁻, SO₄²⁻ ions, and, amyllum, dextrin and glucose will also affect the assays. More importantly, there is a tolerance

level of more than 300 for ascorbic acid and thiamine; more than 100 for riboflavin and pyridoxine. Ligands including CN⁻, SCN⁻, 8-hydroxyquinoline and phenanthroline reduce the CL with tolerable ratio of less than 2. In Song and Hou's work,²⁰ an amount of 0.1 ml of human serum was acidified with 1.0 ml of 0.1 M hydrochloric acid, and was further diluted into 10 ml. This treated samples were then used directly for the determination of vitamin B₁₂ using the conventional luminol-CL assay. In our work, the diluted human serum can be determined directly using mode I and the treated human serum can be determined using mode II. If interferences are presented in the samples, they can be easily eliminated by using an upstream cation exchanger. More importantly, the quantity and activity of vitamin B₁₂ are distinguished features in vitamin B₁₂ determination.¹ The quantity of vitamin B₁₂ is not directly proportional to the activity of vitamin B₁₂. Unfortunately, our system is not able to measure the activity of vitamin B₁₂ yet. Generally, residual analytes can accumulate in the tubing and the flow cell. Regular replacement of these parts is important to ensure analysis reliability with no-cross contamination between samples. Plastic syringes and PMMA microchips offer a disposable option with low replacement and maintenance cost.

High concentrations of unbounded vitamin B₁₂ can be determined directly without treatment by spectrophotometry means (molar extinction coefficient of $27,500 \text{ M}^{-1} \text{ cm}^{-1}$ at 361 nm).²⁸ However, its detection limit is relatively high with the sensitivity of 35 µg/ml.⁷ CL-based assays performed in a FIA and a luminometer can have better sensitivities. Table 3 lists the dynamic ranges, detection limits and analytical times for the current state-of-the art techniques. Our proposed method is comparable with the pioneering work of Qin *et al.*¹⁹ using a similar concept and assay; a similar linear range is also obtained. Comparing our method to the works of other groups (Table 3), our linear ranges are longer with four orders of magnitude for mode I and five orders of magnitude for mode II, though the lower detection ranges are not the lowest. The detection limit of our method is low compared to the other groups. This may be useful for the detection of vitamin B₁₂ but not for analytical purpose. The amount of sample required in our methods for analysis is low and the analytical time is short.

The raw data generated in FIA consists of a series of signal peaks plotted against time. Generally, the base peak or the integral of the peak can be used for measurements. The shape of the peak is affected by the injection rate (manual and automatic). For example, a slow and large sample injection will result in a signal peak with a shorter height and a broader base, and vice versa. Hence, there will be a variation in the results if the same analysis is performed in different laboratories or by different personnel. This problem will not occur with a continuous-flow microfluidic system. The signal is generated continuously, and the signal strength will be constant if the flow rate remains constant. Therefore, the measurement procedure can be standardized.

The determination of vitamin B₁₂ concentration using a CL system is usually performed using a large luminometer or a flow-injection analyzer. Compared to these bulky and heavy instruments, our microfluidic system is small in size and portable. In a micro-flow injection system, samples are injected into a continuous flowing stream of reagents. Thus, a lot of reagents is required. However, the samples and reagents in our system were introduced together simultaneously, and the total amount of reagents used was less than a millilitre. Using a more sensitive PMT sensor would allow further reduction of the device size and sample volume. Although, infusion pumps were used in our experiments, reagents and samples may be infused manually into the device. Alternatively, light and portable infusion pumps or integrated micropumps can be designed and used instead. Analysts are able to bring the device out to the field where samples are obtained for on-the-spot analysis. Being small in size and portable, several of such microfluidic devices may be placed on a single laboratory bench. Hence, parallelization can be achieved in this manner. A continuous-flow syringe infusion pump may be used to continuously feed the device with samples and reagents. More importantly, the low-cost and disposable PMMA microchips can be replaced regularly.

Our reported system can be further modified to provide better functions. A flow injection system can be incorporated into the current system and turned this microfluidic system into an integrated FLA-LOC system to provide automation and quick analysis. An oxidising electrode may be fabricated into the microchip to provide the necessary peroxides for the CL reaction.¹⁹ This method can help to generate ad-hoc H₂O₂ from O₂ and H₂O, and in turn reduces the associated issue with the preparation of H₂O₂. In addition, our system can also be used for other CL assays. For instances, suspended cultured cells can be mixed with a cell lysis buffer, and adenosine triphosphate (ATP) can be detected using luciferase assay in the mixer-detection microchannel network, using mode I. Cells extract after tissue homogenisation can be directly determined with luciferase-ATP assay in mode II. Hence, our microchip designs are not limited by the current CL assay.

Conclusions

The proficiency of a microfluidic CL system for the detection of vitamin B₁₂ was successfully demonstrated. The system was able to detect small amounts of vitamin B₁₂ concentration at picogram level. The LOC device can also determine quantitatively the concentration of vitamin B₁₂ at nanogram and microgram levels. A less corrosive method was also introduced for the acidification of vitamin B₁₂. Determination of vitamin B₁₂ in supplementary tablets and hen egg yolk was successfully demonstrated with this system. The obtained results were in good agreement with the data obtained by UV-visible spectrophotometry. Compared to previously published works and other instrumental systems, our lab-on-a-chip system shows a great potential in providing a low-cost, portable and yet sensitive analysis.

Notes and References

^a National Institute of Education, Nanyang Technological University, 1Nanyang Walk, Singapore, 637616, Singapore. E-mail: peter.lee@nie.edu.sg

^bSchool of Mechanical and Aerospace Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798. Fax: +65 67924062; Tel: +65 67904457; E-mail: mntnguyen@ntu.edu.sg.

1. S. S. Kumar, R. S. Chouhan and M. S. Thakur, *Analytical Biochemistry*, 2010, **398**, 139-149.
2. M. A. Bernard, P. A. Nakonezny and T. M. Kashner, *J Am Geriatr Soc.*, 1998, **46**, 1199-1206.
3. T. S. Hudson, S. Subramanian and R. J. Allen, *J. Assoc. Off. Anal. Chem.*, 1984, **67**, 994-998.
4. R. C. Oh and L. D. Brown, *Am. Fam. Physician*, 2003, **67**, 979-986.
5. E. Miyamoto, Y. Tanioka, T. Nakao, F. Barla, H. Inui, T. Fujita, F. Watanabe and Y. Nakano, *Journal of Agricultural and Food Chemistry*, 2006, **54**, 9604-9607.
6. M. K. Sahni, S. Spanos, M. Z. Wahrman and G. M. Sharma, *Analytical Biochemistry*, 2001, **289**, 68-76.
7. B. Morelli, *J Pharm Sci.*, 1995, **84**, 34-37.
8. P. Moreno and V. Salvadó, *Journal of Chromatography A*, 2000, **870**, 207-215.
9. S. Wongyai, *Journal of Chromatography A*, 2000, **870**, 217-220.
10. P. Vinas, N. Campillo, I. Lopez-Garcia and M. Hernandez-cordoba, *Identification of vitamin B[12] analogues by liquid chromatography with electrothermal atomic absorption detection*, Springer, Wiesbaden, ALLEMAGNE, 1996.
11. H.-B. Li and F. Chen, *Fresenius' Journal of Analytical Chemistry*, 2000, **368**, 836-838.
12. D. Lambert, C. Adjalla, F. Felden, S. Benhayoun, J. P. Nicolas and J. L. Guéant, *Journal of Chromatography A*, 1992, **608**, 311-315.
13. S. A. Baker and N. J. Miller-Ihli, *Spectrochimica Acta Part B: Atomic Spectroscopy*, 2000, **55**, 1823-1832.
14. S. S. Kumar, R. S. Chouhan and M. S. Thakur, *Analytical Biochemistry*, 2009, **388**, 312-316.
15. Y. K. Zhou, H. Li, Y. Liu and G. Y. Liang, *Analytica Chimica Acta*, 1991, **243**, 127-130.
16. N. Akbay and E. Gök, *Journal of Analytical Chemistry*, 2008, **63**, 1073-1077.
17. S. C. Alcock, P. M. Finglas and M. R. A. Morgan, *Food Chemistry*, 1992, **45**, 199-203.
18. Y. Gao, F. Guo, S. Gokavi, A. Chow, Q. Sheng and M. Guo, *Food Chemistry*, 2008, **110**, 769-776.
19. W. Qin, Z. Zhang and H. Liu, *Analytica Chimica Acta*, 1997, **357**, 127-132.
20. Z. Song and S. Hou, *Analytica Chimica Acta*, 2003, **488**, 71-79.
21. L. Marle and G. M. Greenway, *Analytica Chimica Acta*, 2005, **548**, 20-25.
22. Z. R. Xu and Z. L. Fang, *Analytica Chimica Acta*, 2004, **507**, 129-135.
23. Q. Mei, Z. Xia, F. Xu, S. A. Soper and Z. H. Fan, *Analytical Chemistry*, 2008, **80**, 6045-6050.
24. K. S. Lok, Y. C. Kwok and N.-T. Nguyen, *Analyst*, 2011, **136**, 2586-2591.
25. L. Wei, Z. Zhujun and Y. Liu, *Food Chemistry*, 2006, **95**, 693-698.
26. Y. Sun, Y. C. Kwok and N.-T. Nguyen, *J. Micromech. Microeng.*, 2006, **16**.
27. H. A. Lepper, *Official methods of analysis of the Association of official agricultural chemists*, AOAC, 7TH edition, 1950.
28. J. A. Hill, J. M. Pratt and R. J. P. Williams, *Journal of the Chemical Society (Resumed)*, 1964, 5149-5153.
29. H. Becker and C. Gärtner, *Electrophoresis*, 2000, **21**, 12-26.

-
30. T. G. Burdo and W. R. Seitz, *Analytical Chemistry*, 1975, **47**, 1639-1643.
 31. E. E. Ziegler and L. J. Filer, *Present Knowledge in Nutrition, seventh ed*, ILSI Press, Washington, DC, 1996.

5