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Selective extraction and determination of vitamin B_{12} in urine by ionic liquid-based aqueous two-phase system prior to high-performance liquid chromatography

Paula Berton [a,b], Romina P. Monasterio [b,c], Rodolfo G. Wuilloud [a,b,*]

[a] Laboratory of Analytical Chemistry for Research and Development (QUIANID), Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, Padre J. Contreras 1300, Parque Gral. San Martín, M5502JMA Mendoza, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
[c] Departamento de Química, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Santa Rosa, La Pampa, Argentina

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ABSTRACT

A rapid and simple extraction technique based on aqueous two-phase system (ATPS) was developed for separation and enrichment of vitamin B_{12} in urine samples. The proposed ATPS-based method involves the application of the hydrophilic ionic liquid (IL) 1-hexyl-3-methylimidazolium chloride and K_2HPO_4. After the extraction procedure, the vitamin B_{12}-enriched IL upper phase was directly injected into the high performance liquid chromatography (HPLC) system for analysis. All variables influencing the IL-based ATPS approach (e.g., the composition of ATPS, pH and temperature values) were evaluated. The average extraction efficiency was 97% under optimum conditions. Only 5.0 mL of sample and a single hydrolysis/deproteinization/extraction step were required, followed by direct injection of the IL-rich upper phase into HPLC system for vitamin B_{12} determination. A detection limit of 0.09 μg mL^{-1}, a relative standard deviation (RSD) of 4.50% ($n=10$) and a linear range of 0.40–8.00 μg mL^{-1} were obtained. The proposed green analytical procedure was satisfactorily applied to the analysis of samples with highly complex matrices, such as urine. Finally, the IL-ATPS technique could be considered as an efficient tool for the water-soluble vitamin B_{12} extraction.

1. Introduction

Vitamin B_{12} (cyanocobalamin) is an essential nutrient formed by a tetrapyrrole complex, which contains a cobalt (Co) atom in its molecule [1]. Vitamin B_{12} (VB_{12}) promotes growth and cell development, helps in maintenance of the myelin sheath, and supports normal metabolism of fat and carbohydrate in mammals [2]. Moreover, its deficiency may result in anemia, while its prolonged deficiency leads to nerve degeneration and irreversible neurological damage [2]. The human requirements of VB_{12} are about 0.40–2.80 μg per day [3]. The non-vegetarian average diet generally contains adequate daily intake of VB_{12}. However, since plants cannot synthesize VB_{12}, strict vegetarians (vegans) have a greater risk of developing VB_{12} deficiency and, hence, depend on VB_{12}-fortified foods or VB_{12}-containing dietary supplements to meet the requirements [4].

Consequently, a high demand for rapid, specific, and simple methodologies to determine vitamins is growing because of their importance for health [5]. It is clear that determination of trace amounts of VB_{12} in biological samples plays an important role in the fields of medicine and toxicology. In the simplest case, VB_{12} is determined as total Co, assuming that no free Co exists. However, the applicability of these methods for VB_{12} quantitation in real samples is somewhat limited since these methods cannot distinguish between free inorganic Co and Co bonded to VB_{12} forms. More specific, cumbersome and time-consuming analytical systems for VB_{12} detection, including microbiological-, radioisotopic dilution- or enzyme-linked immunosorbent-assays and different methods such as chromatographic, electrochemical, spectroscopic and chemiluminescence were critically reviewed recently by Kumar et al. [6]. Although these methods show some advantages, at the same time they present certain drawbacks such as being laborious, time-consuming, nonspecific, less safe, too expensive and limited sensitivity [6]. Moreover, most of these methods have not been tested in samples with complex matrices, such as urine [7]. One of the most widely used instrumental techniques for VB_{12} separation is high performance liquid chromatography (HPLC) with various detection methods, including UV/Vis [8–13],

* Corresponding author at: Laboratory of Analytical Chemistry for Research and Development (QUIANID), Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, Padre J. Contreras 1300, Parque Gral. San Martín, M5502JMA Mendoza, Argentina. Tel.: +54 261 5244064; fax: +54 261 5244001.
E-mail address: rwuilloud@mendoza-conicet.gob.ar (R.G. Wuilloud).

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atomic absorption spectrometry [14], fluorescence [15,16], or mass spectrometry [17–21]. However, and despite UV/Vis is the most common detector for LC, it shows limited sensitivity and selectivity, making it unsuitable for determining low levels of VB_{12} in presence of complex matrices [9]. Therefore, an extraction technique is usually needed for trace analysis prior to HPLC.

Conventional liquid–liquid extractions (LLE) can effectively decrease detection limits and eliminate matrix interference. However, as an attempt to miniaturize and to overcome some shortcomings originated from LLE, such as limited enrichment factors, slow and tedious procedures, and the use of large volumes of organic solvents; several liquid phase microextraction (LPME) techniques have recently emerged [22,23]. Furthermore, in order to solve safe and environmental problems related to regular organic solvents, their replacement by ionic liquids (ILs) has been proposed. ILs have beneficial properties compared to organic solvents like nonflammability and no detectable vapor pressure [24]. During extraction processes, hydrophobic ILs can lead to obtain IL/water biphasic systems. Compared with hydrophilic ILs, hydrophobic ILs are more expensive, and their number is much more limited than the former ones. Moreover, the high viscosity of the IL phase could induce possible denaturation during extraction/separation of biomolecules using simple IL/water biphasic systems [25]. To overcome these limitations, hydrophilic ILs have been employed in aqueous two-phase systems (IL-ATPSs) for extraction of analytes in the presence of inorganic salts [26]. During ATPS, a dispersion occurs, thus generating a high interfacial contact area between the two phases for efficient mass transfer. Thus, and due to the high water content within the phases (70–90%) and low surface tension between them, ATPS offers a mild and biocompatible method for biomolecules purification [27]. State-of-the-art techniques based on IL-ATPS have been recently proposed as an attractive alternative to conventional extraction procedures for high recovery and purification of several biomolecules [28]. To the best of our knowledge, IL-ATPS technique has never been applied for extraction of water-soluble vitamins during sample preparation steps.

In the present work, a fast and simple clean-up and separation method for selective and accurate VB_{12} determination at trace levels is proposed. VB_{12} was extracted from pre-treated samples by application of ATPS technique based on the IL 1-hexyl-3-methylimidazolium chloride ($[C_6mim][Cl]$) and an inorganic salt. The upper IL enriched phase was directly injected into HPLC for VB_{12} determination. The study focused on the development of an easy, inexpensive, fast, and accurate method for quantitative determination of VB_{12} in biological samples, such as urine.

2. Material and methods

2.1. Instrumentation

Absorbance at 300–600 nm of the stock solutions was scanned with a Lambda 35 UV/Vis spectrometer (Perkin Elmer, Shelton, CT, USA), equipped with 1 cm quartz cuvette. Samples were run in a HPLC system (200LC series, Perkin Elmer), composed by quaternary pump, column oven and UV–VIS detector. The column temperature was maintained at 25 °C and an injection volume of 20 μL was used in all experiments. Chromatographic separations were performed on a Zorbax-SB-Aq column (4.6 × 150 mm, particle size 5 μm), purchased to Agilent Technologies (Santa Clara, CA, USA). The column was run with different mobile phases at a flow rate of 1 mL min^{-1} for 20 min and monitored at 360 nm. Instrumental conditions are summarized in Table 1.

A vortex model Bio Vortex V1 (Boeco, Hamburg, Germany) was used for mixing the phases.

2.2. Reagents

A 1000 mg L^{-1} VB_{12} stock standard solution was prepared by dissolving 10 mg of VB_{12} (Sigma, Milwaukee, WI, USA) in 10 mL of ultrapure water. Lower concentrations were prepared by diluting the stock solution with ultrapure water. Salts evaluated for ATPS occurrence included: KH_2PO_4, KOH, K_2HPO_4, K_2SO_4 and K_2CO_3 and were purchased from Sigma. Different ILs including, $[C_nmim][Cl]$ (n=4, 6, 8) were synthesized according to a method proposed by J.G. Huddleston and coworkers [29]. Qualitative analysis of synthesized IL was performed by comparison of infrared spectra

Table 1
Instrumental and experimental conditions for vitamin B_{12} determination.

Instrumental conditions			
Wavelength	360 nm		
Injection volume	20 μL		
LC column	Zorbax SB-Aq (5 μm × 4.6 mm i.d. x 150 mm)		
Guard column	Zorbax Realiance Analytical Cartridge		
Flow rate	1 mL min^{-1}		
Column temperature	25 °C		
Mobile phases	B: 5 mmol L^{-1} phosphate buffer (pH 5) D: methanol		
Extraction conditions			
Pre-treated sample volume	5.0 mL		
IL amount	0.2 g		
Salt amount (K_2HPO_4)	3.0 g		
Extraction time	5 min		
HPLC gradient program			
Step	Initial time (min)	Final time (min)	Final composition of mobile phase (linear gradient)
0	0.0	0.5	90% B; 10% D
1	0.5	15	10% B; 90% D
2	15	20	10% B; 90% D

with commercially available [$C_n$mim][Cl] (Solvent Innovation GmbH, Köln, Germany).

Ultrapure water (18 MΩ cm) was obtained from a Millipore Continental Water System (Bedford, MA, USA). All glassware was washed with 0.1 mol L^{-1} HNO_3 solution at least for 24 h and thoroughly rinsed 5 times with ultrapure water before any use.

2.3. Sample collection and conditioning

The urine samples used in the analysis were the first-voided morning specimens collected from volunteers. The samples were collected in clean, acid-washed, amber glass bottles. The specimens were centrifuged for 5 min at 2500 rpm (503 g) to remove any insoluble material. The supernatant was transferred to a 20-mL vial for immediate analysis.

2.4. IL-ATPS procedure

An amount of 0.2 g of [C_6mim][Cl] was added and fully dissolved into 5 mL of the pre-treated sample. After addition of 3.0 g of K_2HPO_4, the mixture was vortexed and the homogeneous solution became cloudy, extracting VB_{12} into the IL phase. After 5 min without vortex-assisted stirring, two well-defined phases were formed, without the need of centrifugation. The upper IL-enriched phase was then directly injected into the HPLC column for VB_{12} separation and determination. Calibration was performed by spiking the samples with known concentrations of VB_{12}. Same procedure as described above for samples was applied for calibration standards. Optimized instrumental and experimental conditions are shown in Table 1.

3. Results and discussion

3.1. [C_6mim][Cl]-salt IL-ATPS extraction

Several K^+ ion-containing compounds, including K_2SO_4, KOH, K_2CO_3, K_2HPO_4 and KH_2PO_4 have been evaluated for their suitability to form [C_6mim][Cl]-salts IL-ATPS. Results show that IL-ATPS can be formed only by adding appropriate amounts of K_2CO_3, KOH or K_2HPO_4 to water–[C_6mim][Cl] homogeneous solution. As expected, the observed order follows the Hofmeister series about the strength of kosmotropic salts: $K_2HPO_4 > K_2CO_3 > KOH$. The kosmotropic ions, e.g. HPO_4^{2-}, SO_4^{2-}, OH^-, CO_3^{2-} and PO_4^{3-}, which are usually small and highly charged, exhibit stronger interaction with water molecules than that between water molecules, being beneficial to ATPS formation. However, the chaotropic ions, e.g. Cl^-, $H_2PO_4^-$, which are large-size and lowly charged species, have the opposite effect because of their weaker interactions with water. This theory leads to the statement that ion specificity is mainly determined by the ion's polarizability in water, and the generally observed greater and dominating Hofmeister effects of anions over that of cations can be explained by the larger polarizability values of anions with respect to cations [30,31]. Although SO_4^{2-} has almost the same kosmotropic level as HPO_4^{2-}, the solubility of the former anion is much lower than the second one (11 vs. 150 g/100 mL H_2O). Therefore, SO_4^{2-} concentration can never be high enough to allow the formation of ATPSs, even in saturated solutions. As expected, strongly kosmotropic ions favor IL-ATPS formation, thus less salt amount was needed as more kosmotropic was the assayed salt. However, the mechanism through which the salt influences phase separation is still poorly understood. An appropriate explanation for phase separation in IL-ATPS correlates the observed behavior to the tendency of chaotropic salts to be salted-out by kosmotropic salts. Since ILs are designed to have depressed melting points, a result of low symmetry ions, delocalized charge, and weak directional intermolecular interactions, most ILs would be classified as chaotropic salts [32]. The phase separation process can thus be hypothesized as follows: after addition of an inorganic salt to an IL solution, the ions will compete with each other for the solvent molecules. The more kosmotropic inorganic ions have a stronger affinity for the solvent. Consequently, a "migration" of solvent molecules away from the ions of the IL to those of the inorganic salt takes place, which in turn decreases the hydration and hence the solubility of the ions of the IL. As a consequence, an IL-rich phase separates from the rest of the solution. Therefore, the resulting salting-out effect could be directly correlated to hydration strength of the different ions of inorganic salt [33].

After studying the effect of different salts on IL-ATPS formation, recovery of VB_{12} in the top phase of [$C_n$mim]Cl–salt IL-ATPS was evaluated. The results indicated that K_2HPO_4 led to achieving the highest extraction efficiency. When K_2HPO_4 was employed, a final pH of 11 was obtained. Since VB_{12} is stable within the pH interval of 4.0–12.0 [34], its extraction efficiency could be considered as pH-independent under this interval. Therefore, as expected, the highest extraction efficiency was obtained with K_2HPO_4 because less salt amount was needed for a complete phase separation. Thus, K_2HPO_4 was selected as the phase forming salt. Phase diagrams of IL-K_2HPO_4 systems based on the three alkylimidazolium chloride ILs with different alkyl groups were not determined in this work, since this phase-forming studies were already published in a previous work by Cao et al. [35].

The effect of the amount of K_2HPO_4 on phase behavior of ATPS was also investigated. Different amounts of K_2HPO_4 were added (between 2.0 and 4.0 g) into 5.0 mL water containing 0.2 g IL for the phase separation. The higher was K_2HPO_4 concentration, more [C_6mim]Cl was driven into the upper phase resulting in the decreasing of phase ratio. As a result, the phase separation step was easier and the enrichment factor was optimal. However, when the amount of K_2HPO_4 was higher than 3.0 g, the reached top phase volume was constant at 0.25 mL as the salting-out ability of K_2HPO_4 was maximum. Therefore, 3.0 g of K_2HPO_4 were chosen for further experiments.

Alkyl-imidazolium-ILs with different carbon chains were evaluated in this work. In good agreement with a previous work [30], the phase-forming ability follows the order: $[C_6mim]^+ > [C_4mim]^+ > [C_8mim]^+$. In traditional ATPS, when increasing the molar mass of polyethylene glycol (PEG), the binodal curves became closer to the origin [36]. A suitable explanation could be that the higher hydrophobic character of PEG, accounted for its larger molar mass, increases the incompatibility between the phase-forming components. However, the phase-forming ability of the ILs with different alkyl-chain lengths was not in agreement with the order of their hydrophobicity, i.e. $[C_6mim]^+$ showed the best phase-forming ability. Although this anomalous behavior was previously reported [30,37], the reason has not been fully understood yet.

Since extraction efficiency and analyte detection in HPLC can be remarkably affected by IL amount, this is a critical parameter to be optimized in order to yield the highest VB_{12} extraction while getting the best analytical sensitivity. Recovery of VB_{12} upon different [C_6mim]Cl amounts was examined within the range of 0.1–0.7 g. As shown in Fig. 1, the highest recovery was achieved when 0.1–0.2 g of [C_6mim]Cl was employed. Higher amounts of IL did not improve extraction efficiency, while led to a decrease in the enrichment factor. Therefore, in order to economize IL and gain a higher enrichment factor with minimal matrix effect, 0.2 g was used for subsequent experiments in this work.

The effect of temperature on VB_{12} extraction efficiency was also investigated. Within 10–70 °C, the extraction efficiency remains practically constant, indicating that temperature has little influence on the distribution and kinetic behavior of VB_{12} between the two phases formed. This new extraction system can afford a relatively

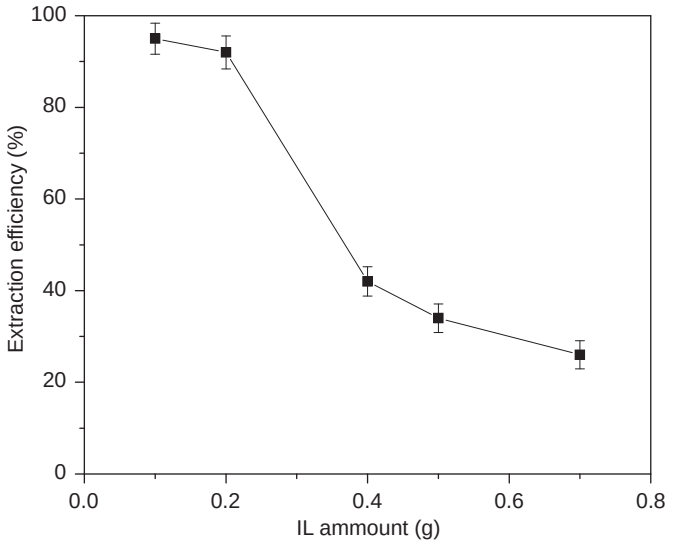

Fig. 1. Effect of IL amount on VB_{12} recovery. Other experimental conditions are illustrated in Table 1.

wide range of temperature for the study on the extraction behavior of VB_{12}. The following studies were developed at room temperature.

3.2. Optimization of separation conditions

Initial studies demonstrated that isocratic elution was suitable for VB_{12} elution from the column when an aqueous standard was injected into a mobile phase containing 40% of 0.005 mol L^{-1} phosphate buffer (pH 5) prepared in high purity water (mobile phase B) and 60% of methanol (mobile phase D) at a flow rate of 1 mL min^{-1}. However, when a VB_{12}-containing IL-matrix standard was injected into the column under the isocratic conditions, retention times were significantly modified and VB_{12} was eluted at a time corresponding to the column dead-volume (Fig. 2a). Therefore, different conditions for gradient elution were studied in this work. Improved resolution was achieved when a gradient program was employed (Fig. 2b). After 0.5 min the isocratic run (90% B and 10% D) was started, solvent B was decreased linearly (increasing D) and reached 10% at 15 min. The final composition (10% B and 90% D) was kept constant for 5 min (until 20 min). After the acquisition time, 5 min post time was set for the equilibration of the initial solvent composition. The selected column, a Zorbax SB-Aq, has an alkyl reversed bonded phase designed to retain hydrophilic and other compounds, while it is compatible with the most common mobile phases, including highly aqueous ones. Under these conditions, VB_{12} was eluted within 10 min (Fig. 2b) and no significant interfering peaks were observed at this retention time.

3.3. Analytical performance

The partition of VB_{12} between the phases was characterized by several parameters, including extraction efficiency and enrichment factor (EF). The EF was defined as the ratio of the calibration curve slopes before and after the preconcentration step [38]. Hence, at optimal experimental conditions, the obtained extraction efficiency was 97% and the EF for a sample volume of 5 mL was 25. The relative standard deviation (RSD) resulting from the analysis of 10 replicates of 5 mL solution containing 0.50 μg mL^{-1} VB_{12} was 4.5%. The calibration graph was linear with a correlation coefficient of 0.9979 within a concentration range between 0.40 μg mL^{-1} and up to at least 8.00 μg mL^{-1}. The limit of detection (LOD) of the proposed methodology, calculated based on the signal at intercept and three times the standard deviation about regression of the calibration curve, was 0.09 μg mL^{-1}.

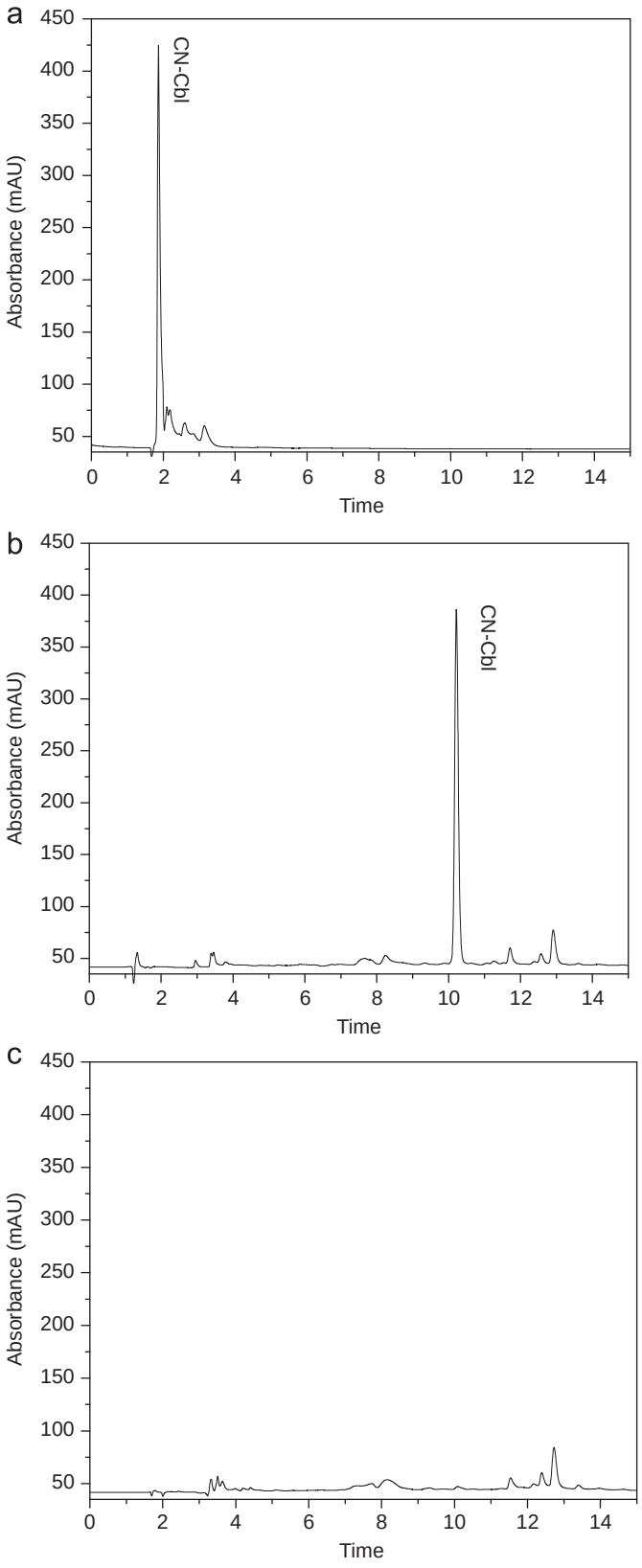

Fig. 2. Chromatogram obtained for (a) VB_{12} standard of 100 μg mL^{-1} in an IL matrix with isocratic elution; (b) VB_{12} standard of 100 μg mL^{-1} in an IL matrix with gradient elution; and (c) IL without VB_{12}. Other experimental conditions are illustrated in Table 1.

Table 2
Determination of vitamin B_{12} in urine samples (95% confidence interval; $n=6$).

Sample	Added ($\mu g\ mL^{-1}$)[a]	Found ($\mu g\ mL^{-1}$)	Recovery (%)[b]
Urine 1	0.00	3.00 ± 0.10	–
	1.00	3.88 ± 0.12	97.0
	3.00	6.36 ± 0.25	106
Urine 2	0.00	[b]	–
	1.00	0.98 ± 0.04	98.1
	3.00	3.10 ± 0.14	103
Urine 3	0.00	0.80 ± 0.07	–
	1.00	1.76 ± 0.05	97.5
	3.00	3.76 ± 0.11	99.1
Urine 4	0.00	[b]	–
	1.00	1.01 ± 0.03	101
	3.00	2.95 ± 0.10	98.3
Urine 5	0.00	[b]	–
	1.00	1.02 ± 0.04	102
	3.00	3.02 ± 0.11	100

[a] $[(\text{Found} - \text{Base})/\text{Added}] \times 100$.
[b] Not detected.

Furthermore, capacity factor, a chromatographic parameter which characterize the performance of the method, was calculated as $k' = (t_R - t_0)/t_0$; where t_R is the migration time and t_0 is the dead time. A capacity factor of 6.68 was obtained with the proposed method. It is widely accepted, that capacity factors between 2 and 10 are optimum in practice for a mixture of few components. Moreover, reproducible retention times were observed throughout a regular working day (8–12 h of analysis).

3.4. Application of IL-ATPS-HPLC method for vitamin B_{12} determination in complex samples

In order to demonstrate the applicability of the proposed method to matrices where VB_{12} determination is highly significant, the proposed method was applied to urine samples (Table 2). Blood is the ideal matrix for most chemicals due to its contact with the whole organism and its equilibrium with organs and tissues where chemicals are stored [39]. However, blood requires an invasive extraction method to be obtained and collected amounts are limited. On the other hand, urine can be collected in larger amounts and by non-invasive methods. Moreover, urine is the second most common matrix for the biomonitoring of water-soluble compounds [40]. The representative chromatogram of urine after IL-ATPS-HPLC analysis is shown in Fig. 3. No evident interference was observed on the separation of VB_{12}. The results showed excellent selectivity of the method for the determination of VB_{12}. Furthermore, high reproducibility of retention time for VB_{12} was obtained in presence of the sample matrix, which might allow the application of the proposed method to samples different than urine.

The analytical recovery of VB_{12} in urine (Table 2) was also studied. The proposed method was applied to six portions of different matrices and average concentrations of VB_{12} were taken as base values. Then, 1.00 and 3.00 $\mu g\ mL^{-1}$ VB_{12} were added to samples and the same procedure was followed. VB_{12} recoveries were highly satisfactory in all cases.

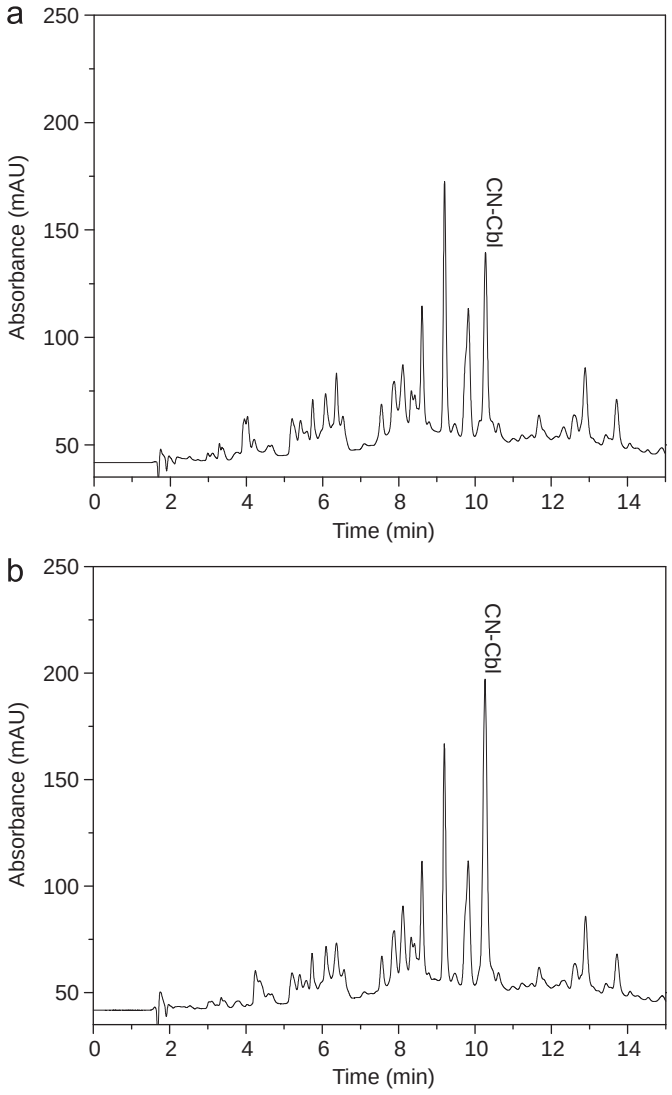

Fig. 3. Determination of VB_{12} in urine sample with IL-ATPS-HPLC method: (a) urine sample 1 without VB_{12} standard addition; (b) urine sample 1 spiked at 2 $\mu g\ mL^{-1}$ of VB_{12}. Experimental conditions are illustrated in Table 1.

Table 3
Performance data of the proposed method compared to other methods with HPLC–UV detection for VB_{12} determination.

Ref.	Extraction time (min)	LOD ($ng\ mL^{-1}$)	RSD (%)	Sample consumption	Calibration range ($\mu g\ mL^{-1}$)
[41]	[a]	40.0	[a]	[a]	2.00–16.0
[42]	25	[a]	2.68–6.79	[a]	0.005–15
[7]	[a]	33.0	0.84	1 tablet	0.01–1.00
[11]	15	97.5	3.20	1 g	0.01–1.00
[43]	[a]	40.0	6.02	[a]	0.04–0.12
Present work	5	90.0	4.50	5 mL	0.40–8.00

[a] Not reported.

4. Conclusions

A rapid microextraction method based on $[C_6mim]Cl$ IL for selective vitamin B_{12} determination is presented in this work. Due to the low solvent consumption and the selection of IL as organic phase, the developed method is in good agreement with green chemistry principles. Compared with conventional ATPS, the method presents many advantages such as low viscosity, quick phase separation and high extraction efficiency. Moreover, the proposed IL-ATPS is simpler and faster, compared to conventional solid-phase extraction (SPE) (which usually includes a number of laborious steps, such as sorbent conditioning, rinsing the sample, washing and elution of the analytes).

All in all, the results shown in this work indicate that the proposed procedure is simple, fast, interference-free, selective and

environment-friendly, and it can be used for VB_{12} selective preconcentration and determination in urine. IL-ATPS technique combined with HPLC chromatography shows a good limit of detection and a wide calibration range with a reduced amount of sample, while using low cost and widely spread instrumentation. The method detection limit is comparable to, or better than, others extraction methods prior HPLC–UV analysis reported for VB_{12} (Table 3). Furthermore, most of the extraction methodologies previously proposed, employed larger volumes of sample and longer extraction times, when informed. The simplicity of the process and the low cost of phase-forming materials make the proposed method feasible for large-scale vitamin purification using appropriate scale-up techniques. The present study demonstrates that IL-ATPS can be an excellent and green extraction technique for separation and preconcentration of water-soluble vitamins, even from complex matrices like urine.

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