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# Selective determination of inorganic cobalt in nutritional supplements by ultrasound-assisted temperature-controlled ionic liquid dispersive liquid phase microextraction and electrothermal atomic absorption spectrometry

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ABSTRACT

In the present work, a simple and rapid analytical method based on application of ionic liquids (ILs) for inorganic Co(II) species (*i*Co) microextraction in a variety of nutrient supplements was developed. Inorganic Co was initially chelated with 1-nitroso-2-naphtol (1N2N) reagent followed by a modern technique named ultrasound-assisted temperature-controlled ionic liquid dispersive liquid phase microextraction (USA-TILDLME). The extraction was performed with 1-hexyl-3-methylimidazolium hexafluorophosphate [$C_6$mim][$PF_6$] with the aid of ultrasound to improve *i*Co recovery. Finally, the *i*Co-enriched IL phase was solubilized in methanol and directly injected into an electrothermal atomic absorption spectrometer (ETAAS). Several parameters that could influence *i*Co microextraction and detection were carefully studied. Since the main difficulty in these samples is caused by high concentrations of potential interfering ions, different approaches were evaluated to eliminate interferences. The limit of detection (LOD) was 5.4 ng $L^{-1}$, while the relative standard deviation (RSD) was 4.7% (at 0.5 μg $L^{-1}$ Co level and $n$ = 10), calculated from the peak height of absorbance signals. Selective microextraction of *i*Co species was achieved only by controlling the pH value during the procedure. The method was thus successfully applied for determination of *i*Co species in nutritional supplements.



## 1. Introduction

It is becoming increasingly recognized that composition of a person's diet is an important determinant in resistance to many adverse factors, including environmental stresses. Nowadays, a wide variety of nutritional supplements recommended for overcoming such tensions are being marketed by companies responsible for these claims [1]. Regulation of these supplements is however complex. Cobalt (Co), an essential trace element in nature, has an important role in many body functions, as a component of vitamin $B_{12}$ [2]. However, unnecessary Co(II) salts administration causes adverse side effects, since its accumulation promotes organ damage and dysfunction due to enhanced oxidative stress. Excessive inorganic Co(II) (*i*Co) in blood impairs thyroid activity and myocardial function, promoting carcinogenesis [3]. Moreover, ingestion of *i*Co at higher amounts leads towards a systemic but reversible allergy termed dyshidrotic eczema [4]. Therefore, determination of Co species, such as vitamin $B_{12}$ and *i*Co, results critical to evaluate benefits and risks of Co consumption originated from nutritional supplements. Electrothermal atomic absorption spectrometry (ETAAS) is the most used analytical technique for trace elements determination in food and related samples, due to its reliability, sensitivity and relatively low cost of instrumentation [5]. Nevertheless, difficulties still lie on determination of trace heavy metals because of both, their low abundance occurring in these samples and high concentrations of potential interfering ions, such as iron or zinc. Hence, preliminary preconcentration and sample clean-up steps are frequently required to achieve accurate, reliable and sensitive results.

Although conventional liquid–liquid extractions (LLE) with regular organic solvents can effectively decrease detection limits and eliminate matrix interferences, they also have several shortcomings such as limited enrichment factors and generally involve slow and tedious procedures. Moreover, the use of large volumes of organic solvents gives rise to large amounts of organic wastes, resulting in environmental and safety concerns due to high volatility, toxicity and flammability [6]. Thus, a clear trend in analytical chemistry is miniaturization of classical sample preparation techniques. In the last years, several liquid phase microextraction (LPME) techniques have emerged as an attempt to miniaturize and

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improve LLE [7]. Thus, ETAAS is commonly coupled with LPME approaches, due to its suitability to work at microvolume scale [8]. On the other hand, many of the problems linked with regular organic solvents, as well as loss of solvent by evaporation, can be significantly avoided using ionic liquids (ILs) as alternative solvents, since they have no detectable vapor pressure and are relatively thermal stable even at elevated temperatures [9]. Extractions of metal ions using ILs combined with suitable complexing agents have been recently developed in analytical chemistry [10]. Due to their relative expensiveness, miniaturization of sample pretreatment protocols based on ILs has been developed. Ionic liquids, such as 1-alkyl-3-methylimidazolium hexafluorophosphates ($[C_n mim][PF_6]$, $n = 4, 6, 8$), have been mainly employed in several microextraction techniques [11]. Since these techniques are surface dependent processes, dispersive liquid–liquid microextraction technique using ILs as extractant phases (IL-DLLME) was recently proposed, thus improving the contact area between sample solution and ILs [12]. Besides classical IL-DLLME with organic solvents as dispersing agents, different alternatives have been developed to obtain a dispersion of the extraction solvent into sample solutions. Thus, increase of temperature or application of ultrasound energy to sample solutions has been employed as dispersant tools in microextraction procedures. Therefore, emerging temperature-controlled IL dispersive liquid phase microextraction (TILDLME) and ultrasound-assisted ionic liquid dispersive liquid–liquid phase microextraction (IL-USA-DLLME) techniques have been developed, respectively [9,10,13].

Several IL-LPME approaches involving metal chelation with pyridylazo-type reagents [14–17], ammonium pyrrolidinedithiocarbamate (APDC) [18], 1-phenyl-3-methyl-4-benzoyl-5-pyrazolone [19] have been reported for Co microextraction. However, these reagents showed limited selectivity towards metal chelation. As a consequence, extraction procedures based on these reagents could be prone to suffer from matrix interferences, such as those occurring in real complex samples. On the other hand, 1-nitroso-2-naphthol (1N2N) can selectively react with *i*Co under specific conditions [20]. Moreover, despite the high number of separation and preconcentration techniques proposed for total Co determination in nutritional samples, there is still need for methods capable of discriminating between *i*Co and Co under vitamin $B_{12}$ species.

In this work, a highly selective separation and preconcentration method for determination of *i*Co species at trace levels is proposed. Inorganic Co species was initially complexed with 1N2N reagent, followed by application of TILDLME technique based on 1-hexyl-3-methylimidazolium hexafluorophosphate ($[C_6mim][PF_6]$) IL and the aid of ultrasound energy to improve *i*Co recovery. The proposed method was successfully applied for the determination of *i*Co at trace levels in several nutritional supplements with different matrices.

## 2. Experimental

### 2.1. Instrumentation

Experiments were performed using a Perkin Elmer (Shelton, CT, USA) model 5100PC atomic absorption spectrometer equipped with a graphite furnace module (HGA 500), a pyrolytic graphite tube (Perkin-Elmer) and a transversely heated graphite atomizer with Zeeman-effect background correction system. A Co hollow cathode lamp (SCP Science, Champlain, NY, USA) operated at a current of 15 mA and a wavelength of 240.7 nm with a spectral bandwidth of 0.2 nm was used. All measurements were performed using integrated absorbance with an integration time of 5 s. Temperature and time programs for ETAAS instrument were as mentioned in Table 1.

**Table 1**
Instrumental and experimental conditions for Co determination.

| Instrumental conditions | |
|---|---|
| Wavelength | 240.7 nm |
| Spectral band width | 0.2 nm |
| Lamp current | 15 mA |
| Injection volume | 80 μL |
| Modifier (Pd) mass | 5 μg Pd |

Graphite furnace temperature program

| Step | Temperature (°C) | Ramp time (s) | Hold time (s) | Argon flow rate ($mL\,min^{-1}$) |
|---|---|---|---|---|
| Drying 1 | 110 | 1 | 30 | 250 |
| Drying 2 | 250 | 15 | 30 | 250 |
| Pyrolysis 1 | 600 | 90 | 30 | 250 |
| Pyrolysis 2 | 900 | 20 | 20 | 250 |
| Pyrolysis 3 | 1200 | 10 | 20 | 250 |
| Atomization | 2400 | 0 | 3 | – |
| Cleaning | 2600 | 1 | 2 | 250 |

| Extraction conditions | |
|---|---|
| Pre-treated sample volume | 5 mL |
| 1N2N concentration | $1.2 \times 10^{-4}\,mol\,L^{-1}$ |
| Working pH | 4.5 |
| Buffer concentration | $1.8 \times 10^{-2}\,mol\,L^{-1}$ |
| Surfactant concentration (Triton X-114) | $1.9 \times 10^{-4}\,mol\,L^{-1}$ |
| RTIL amount | 80 mg |
| Heating temperature | 70 °C (15 min) |
| Extraction time (Cooling and ultrasound) | 3 min |
| Centrifugation time | 10 min ($302 \times g$) |
| IL phase solvent volume | 70 μL |

A centrifuge (Luguimac, Buenos Aires, Argentina) model LC-15 was used to speed up the phase separation process. A thermostated bath (Vicking, Buenos Aires, Argentina) model Masson Digital, maintained at the desired temperature, was used for heating. A vortex model Bio Vortex V1 (Boeco, Hamburg, Germany) was used for mixing the reagents. An ultrasound bath (40 kHz and 600 W) with temperature control (Test Lab, Buenos Aires, Argentina) was used.

### 2.2. Reagents

A 1000 mg $L^{-1}$ Co(II) stock standard solution was prepared by dissolving 0.503 g of Co(II) nitrate hexahydrate (98%) (Aldrich, Milkwaukee, WI, USA) in 100 mL of $1.5 \times 10^{-2}$ mol $L^{-1}$ $HNO_3$ (Merck, Darmstadt, Germany). Lower concentrations were prepared by diluting the stock solution with a $10^{-3}$ mol $L^{-1}$ $HNO_3$ solution. A $10^{-2}$ mol $L^{-1}$ 1N2N solution was prepared by dissolving 0.177 g of 1N2N (98%) (Aldrich) in 100 mL of methanol (Merck). Lower concentrations were prepared by serial dilution with methanol. A 2.0 mol $L^{-1}$ acetic acid–acetate solution (Merck) adjusted to pH 4.5 by dissolution of sodium hydroxide (Merck) was employed as buffer solution. Surfactant solution containing $1.9 \times 10^{-2}$ mol $L^{-1}$ Triton X-114 (Merck) was employed as anti-sticking agent. For chemical modification, a 1 g $L^{-1}$ Pd solution was prepared by dissolving 62.73 mg $Pd(NO_3)_2{\cdot}2H_2O$ (Fluka, Buchs, Switzerland) in 25 mL of a $1.5 \times 10^{-2}$ mol $L^{-1}$ $HNO_3$ solution. A 40 g $L^{-1}$ NaF solution was prepared by dissolving 4 g of NaF (Fluka, Darmstadt, Germany) in 100 mL of ultrapure water.

$[C_6mim][PF_6]$ was synthesized according to a method proposed by Huddleston et al. [21] and stored in contact with ultrapure water to equilibrate the water content in the IL phase [22]. Qualitative analysis of synthesized IL was performed by comparison of infrared

**Table 2**
Nutritional composition of analyzed samples.

| Sample | Form | Composition (nutritional information declared) |
|---|---|---|
| 1[a,b] | Tablet | Vitamins: $B_1$ (11.1 mg g$^{-1}$), $B_2$ (11.1 mg g$^{-1}$), $B_3$ (5.55 mg g$^{-1}$), $B_5$ (5.55 mg g$^{-1}$), $B_6$ (11.1 mg g$^{-1}$), $B_8$ (333.3 μg g$^{-1}$), $B_9$ (444.4 μg g$^{-1}$), $B_{12}$ (5.55 μg g$^{-1}$), C (33.3 mg g$^{-1}$), E (6.67 mg g$^{-1}$). Minerals: Zinc (5.55 mg g$^{-1}$), Selenium (22.2 μg g$^{-1}$) Magnesium (111.1 μg g$^{-1}$), Iron (18.4 mg g$^{-1}$). Ginkgo Biloba (leaves): 22.2 mg g$^{-1}$; Ginseng (powder roots): 55.5 mg g$^{-1}$; Guarana: 55.5 mg g$^{-1}$. |
| 2[c] | Tablet | *Spirulina máxima* and *Spirulina platensis* |
| 3[d] | Capsules | Brewer's yeast with vitamin $B_9$, zinc and magnesium |
| 4[a] | Tablet | Vitamins: $B_1$ (3 mg g$^{-1}$), $B_2$ (3.4 mg g$^{-1}$), $B_3$ (14 mg g$^{-1}$), $B_6$ (4 mg g$^{-1}$), $B_8$ (30 μg g$^{-1}$), $B_9$ (400 μg g$^{-1}$), $B_{12}$ (6 μg g$^{-1}$), C (120 mg g$^{-1}$), E (10 mg g$^{-1}$), A (600 μg g$^{-1}$), K (65 μg g$^{-1}$), $D_2$ (5 μg g$^{-1}$). Minerals: Calcium (250 mg g$^{-1}$), Copper (800 μg g$^{-1}$), Iron (14 mg g$^{-1}$). |
| 5[a] | Solution (beverage) | Vitamins: $B_1$ (1.55 μg g$^{-1}$), $B_2$ (1.75 μg g$^{-1}$), $B_3$ (20.4 μg g$^{-1}$), $B_5$ (10.2 μg g$^{-1}$), $B_6$ (2.04 μg g$^{-1}$), $B_8$ (0.31 μg g$^{-1}$), $B_9$ (0.41 μg g$^{-1}$), $B_{12}$ (6.13 ng g$^{-1}$), Choline (0.41 mg g$^{-1}$), C (0.12 mg g$^{-1}$), E (20.4 μg g$^{-1}$), A (1.53 μg ER g$^{-1}$), D (0.01 μg g$^{-1}$), K (0.08 μg g$^{-1}$). Minerals: Sodium, (0.98 mg g$^{-1}$), Potassium (1.8 mg g$^{-1}$), Calcium (0.82 mg g$^{-1}$) Iron (0.02 mg g$^{-1}$), Phosphor (0.82 mg g$^{-1}$), Iodine (0.15 μg g$^{-1}$) Zinc (15.5 μg g$^{-1}$), Selenium (0.07 μg g$^{-1}$) Magnesium (0.41 mg g$^{-1}$), Copper (2.04 μg g$^{-1}$), Manganese (5.32 μg g$^{-1}$), Chromium (0.12 μg g$^{-1}$), Molybdenum (0.15 μg g$^{-1}$), Chloride (1.84 mg g$^{-1}$). |
| 6[a] | Solution (parenteral nutrition) | Vitamins: $B_1$ (1.66 mg g$^{-1}$), $B_2$ (1.07 mg g$^{-1}$), $B_3$ (8.30 mg g$^{-1}$), $B_6$ (0.41 mg g$^{-1}$), $B_{12}$ (24.90 μg g$^{-1}$). Minerals: Calcium (14.19 mg g$^{-1}$) Iron (4.56 mg g$^{-1}$), Zinc (0.13 mg g$^{-1}$), Magnesium (0.41 mg g$^{-1}$), Manganese (0.13 mg g$^{-1}$). |

[a] Product synthetic, containing vitamins, minerals.
[b] Product containing plant extracts.
[c] Product based on algal extracts.
[d] Product based on yeast extracts.

spectra with commercially available $[C_6mim][PF_6]$ (Solvent Innovation GmbH, Köln, Germany).

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

### 2.3. Sample collection and conditioning

Samples of a variety of commercially available supplements marketed in Argentina, showing different matrices, were selected and prepared in duplicate. Products containing vitamins and/or minerals, under different dosage forms, such as tablets, capsules and, oral and parenteral solutions were analyzed. Detailed information, on analyzed dietary supplements including, classification, dosage forms and nutritional information are presented in Table 2. Solid samples were homogenized by grinding. The contents of capsules and liquid samples were used as it is for following steps. Samples were weighed (average sample weight: 0.5 g), mixed with 6 mL of water, and then shaken on a vortex stirrer. Later on, and after 30 min of metal extraction in an ultrasonic bath, the sample tubes were centrifuged for 10 min at 2500 rpm (503 × *g*). A portion of the supernatant (5 mL) was taken and mixed with 5 mL of a 40 g L$^{-1}$ NaF solution. After 15 min, the solution was centrifuged 10 min at 2500 rpm (503 × *g*). A 5 mL-fraction of the supernatant was then utilized for developing of ultrasound-assisted TILDLME (USA-TILDLME).

### 2.4. Ultrasound-assisted TILDLME procedure

A mixture of 5 mL of the pre-treated sample (for optimization, 1 μg L$^{-1}$ Co(II) standard solution was added to pre-treated samples), 600 μL of $10^{-3}$ mol L$^{-1}$ 1N2N solution, 45 μL of 2 mol L$^{-1}$ (pH 4.5) acetate/acetic acid buffer and 50 μL of $1.9 \times 10^{-2}$ mol L$^{-1}$ Triton X-114, was heated in a thermostated bath at 70 °C for 15 min. After formation of Co–1N2N complex, 80 mg of $[C_6mim][PF_6]$ were added and fully dissolved into the aqueous phase. The homogeneous solution was then placed in an ultrasound bath at a temperature lower than 10 °C for 3 min. A cloudy solution was immediately formed, extracting the Co–1N2N complex into the IL phase. Finally, centrifugation at 1500 rpm (302 × *g*) for 10 min allowed the formation of two well-defined phases. The upper aqueous phase solution was then manually removed with a syringe while the IL phase was dissolved with 70 μL of methanol, followed by direct injection into the graphite furnace for Co determination (Table 1). Calibration was performed against aqueous standards and blank solutions analyzed under the same procedure as described above.

## 3. Results and discussion

Since typical variables affecting complex formation and stability such as pH and acetic/acetate buffer solution concentration were already studied in a previous work [20], these variables were not re-evaluated in the present study (Table 1). On the other hand, the current work was focused on studying critical variables for selective *i*Co extraction and preconcentration from complex samples. Moreover, different IL-based microextraction approaches for high *i*Co recovery were developed and critically compared.

### 3.1. Optimization of microextraction procedure

Development and application of different DLLME procedures were considered to achieve the highest *i*Co extraction recovery. Accordingly, DLLME, TILDLME and USA-TILDLME were evaluated. Despite its simplicity, rapidity and cost effectiveness, DLLME still needs a third component (an organic solvent as disperser) which could decrease the partition coefficient of analytes into the extractant solvent [9]. On the other hand, extraction and preconcentration of compounds are much safer with TILDLME technique, since only small amounts of surfactant (anti-sticking agent) and IL are used. Moreover, the contact area between sample and extractant is dramatically increased [9]. The application of ultrasound energy to sample solutions was evaluated in this work with the aim of increasing extraction efficiency attained with TILDLME technique. As shown in Fig. 1, the highest *i*Co recovery was obtained with USA-TILDLME technique due to smaller droplet size of the dispersed IL phase upon application of ultrasound energy. Thus, submicron droplet-size results in significant enlargement of contact interfacial surface between both immiscible liquids, improving mass-transfer between the immiscible phases, which leads to an increment in the extraction efficiency of the procedure in a minimum time [23].

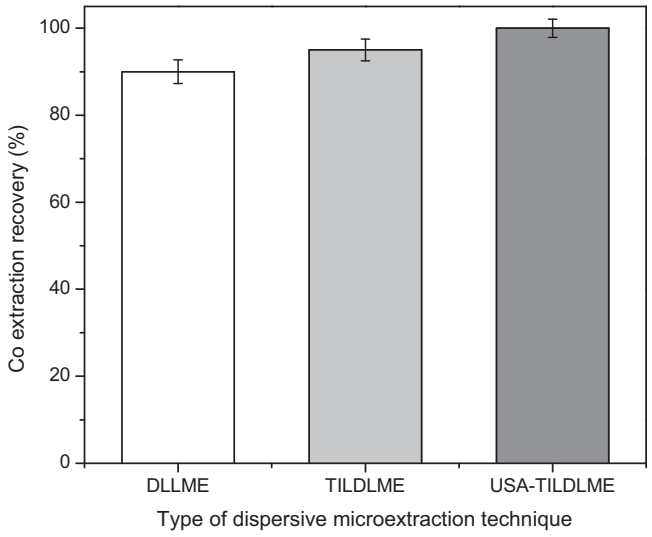


**Fig. 1.** Evaluation of different IL-based dispersive liquid–liquid microextraction techniques on *i*Co recovery (%).

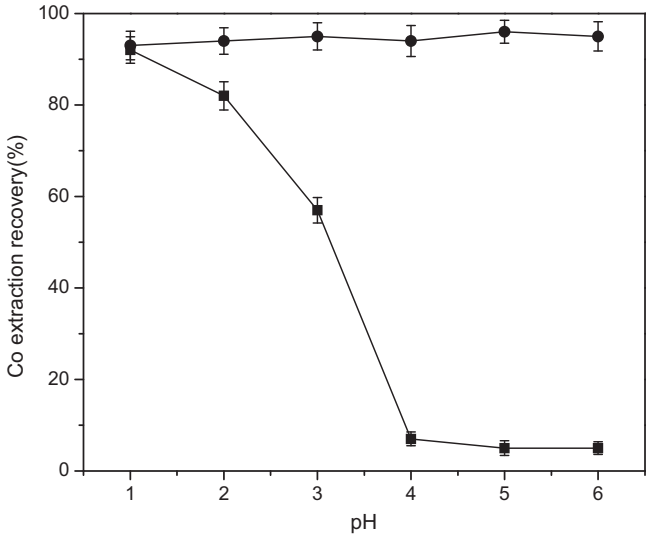


**Fig. 2.** Effect of pH on selectivity and separation of Co species. Inorganic Co (●); Co as vitamin $B_{12}$ (■), both at 0.5 μg $L^{-1}$ Co concentration. Other conditions were as indicated in Table 1.

In order to select a suitable IL, some physicochemical properties were initially evaluated. In this work, we focused on hydrophobic ILs containing imidazolium cation and $PF_6^-$ anion. Considering its high density, acceptable viscosity and low water solubility [24], [$C_6$mim][$PF_6$] IL was chosen as the extractant phase as it forms biphasic systems with aqueous solutions.

Extraction efficiency and analyte detection in ETAAS can be remarkably affected by IL amount. Therefore, this critical parameter was optimized in order to achieve total *i*Co extraction and the highest analytical sensitivity. Recovery of *i*Co upon different amount of [$C_6$mim][$PF_6$] IL was examined within the range of 40–90 mg. The results revealed that 80 mg was the minimal amount of [$C_6$mim][$PF_6$] IL required to obtain a recovery higher than 90%. Higher amounts of IL did not improve extraction efficiency, while increased background signals. Thus, 80 mg was used for subsequent experiments in this work.

In order to improve affinity of *i*Co for the IL phase, 1N2N was selected as chelating reagent. The reaction between *i*Co and 1N2N proceeds rather slowly. Therefore, to speed up the reaction and assure Co–1N2N formation since low *i*Co concentrations, an excess of 1N2N reagent should be used. The effect of 1N2N concentration on the analytical signal was evaluated in presence of concomitant ions which could be frequently found in the samples under study. The highest *i*Co recovery was observed at $1.2 \times 10^{-4}$ mol $L^{-1}$ 1N2N concentration, while a drop of extraction efficiency was observed for higher concentrations. This effect was also observed in a previous work [20], and it was attributed to an increase of hydrophobic IL solubility in acidic aqueous phase induced by the presence of a neutral extractant, such as 1N2N. Therefore, the protonate form of the extractant, generated in the acidic medium, may exchange with the cationic component of the IL, resulting in an increase of the solubility of the IL [25].

The adherence of the IL on the inner walls of plastic centrifuge tubes was observed. A non-ionic surfactant was added to the solutions to reduce this effect due to the ability of its molecules for surrounding the fine droplets of IL. Hence, interactions of IL with the inner walls of the centrifuge tubes decrease. In a previous work, Triton X-114 was selected as the best anti-sticking agent [20]. The effect of different concentrations ($0–3.7 \times 10^{-4}$ mol $L^{-1}$) was studied. Inorganic Co extraction improved up to $1.9 \times 10^{-4}$ mol $L^{-1}$. Therefore, this concentration was employed for further experiments. The use of IL-based microextraction techniques for the treatment of samples with high content of electrolytes is usually problematic since solubility of some IL in water dramatically depends on ionic strength. At high ionic strength values, the IL is completely soluble in water and its recovery after extraction is unaffordable [9]. Therefore, synthetic samples containing salts concentrations emulating real samples were assayed for salt effect during microextraction. No visible effect was observed for *i*Co recovery when same concentrations of salts than those occurring in samples were applied to synthetic solutions.

Extraction is a time-dependent process which involves transferring of analytes from aqueous into IL phase. The extraction time, defined as the interval between the solution was placed into the cold bath under the influence of ultrasound and the time that centrifugation was started, was evaluated in the range of 0–20 min. The recovery-time study showed that the highest extraction efficiency could be attained in 3 min only, while longer extraction times did not significantly improve the performance of the procedure. These results show a very fast extraction process, as soon after the cloudy solution is formed; the interfacial area between the IL droplet and the aqueous phase is very large, thus significantly increasing the diffusion of *i*Co–1N2N complex into the extractant. In order to achieve the highest extraction efficiency in the shortest time, extraction was performed during 3 min. After extraction time, and due to the relatively high stability of the IL-emulsion, a centrifugation step was required. Different centrifugation times were assayed at a constant speed of 1500 rpm ($302 \times g$). Higher speeds compromised the structural integrity of glass centrifugation tubes. Only 10 min of centrifugation were necessary for complete phase separation.

### 3.2. Selective microextraction of inorganic Co

Selective microextraction of *i*Co was evaluated upon different pH values of sample solutions. It was observed that both *i*Co and vitamin $B_{12}$ were at low pHs only (Fig. 2). In fact, extraction of Co contained in vitamin $B_{12}$ was feasible even without 1N2N addition. This phenomenon could be attributed to chemical alteration of vitamin $B_{12}$, as this compound has functional groups susceptible to suffer a variety of chemical modifications depending on temperature and acid concentration, among others [26]. On the other hand, once formed, *i*Co–1N2N complex could be extracted at any acidic pH due to its high stability [27]. Thus, complete separation of Co species was achieved at pHs higher than 4, Therefore, a simple

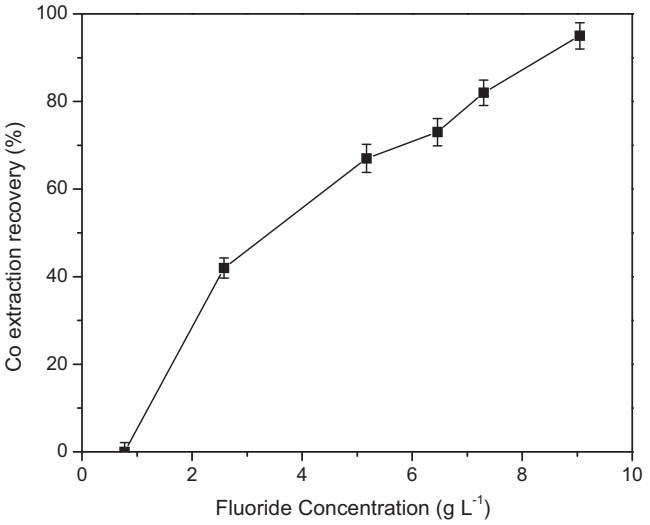


**Fig. 3.** Selectivity of *i*Co complex formation and extraction: effect of fluoride ion concentration on *i*Co recovery (%). Other conditions were as indicated in Table 1.

approach based on right selection of pH was pursued for selective extraction and determination of *i*Co.

### 3.3. ETAAS conditions for Co determination in IL phase

Since a complete study on Co measurement by ETAAS in the presence of IL matrix was already developed in a previous work [20], the same chemical modifier and graphite furnace program (Table 1) were applied in this study. In order to achieve reproducible injection of the IL into the graphite furnace, methanol was chosen for IL-phase dilution. In the previous work, it was observed that the best analytical performance was obtained when methanol was employed as diluent [20]. Furthermore, the volume of methanol was a critical variable to be assayed. Total dissolution of the IL phase was observed for 70 μL methanol, while smaller volumes turned out into deleterious effects on analytical sensitivity. Thereby, 70 μL of methanol was employed for further experiments. The entire resulting methanol-IL phase was injected and successfully analyzed by ETAAS under the conditions showed in Table 1.

### 3.4. Study on potential interfering species

A very useful strategy to eliminate interferences was initial formation of metal–naphtolate complexes at pH 4, followed by decreasing of pH to 1, thus decomposing all metal-complexes except *i*Co–1N2N. However, considering the high concentration of concomitant ions occurring in some of the samples, a high concentration of 1N2N was required, thus increasing solubilization of IL into the aqueous phase, as explained in Section 3.1. Also, as mentioned in Section 3.2, at low pHs, vitamin $B_{12}$ becomes extractable into IL phase. Therefore, another strategy had to be adopted. Since Fe and Zn could interfere more seriously than any other ions, addition of NaF as masking agent was studied to correct for any interferent effects. In fact, these elements show the highest concentrations in nutritional supplement samples (Table 2). A study of the minimal fluoride concentration necessary to mask concomitants ions present in these samples was developed. Fig. 3 shows that a minimal fluoride concentration of 9.05 g $L^{-1}$ was necessary to obtain *i*Co recovery higher than 90%. Furthermore, the selectivity of the proposed method was assayed by evaluating the effect of possible concomitant ions at the levels usually found in nutritional supplements. The procedure was performed with 5 mL of 1 μg $L^{-1}$ *i*Co solution containing different concentrations of such ions. As shown in Table 3, quantitative separation and determination of *i*Co were obtained in presence of foreign ions and at concentrations normally found in the samples under study.

**Table 3**
Effect of foreign ions on the recovery of *i*Co.[a]

| Ion | Added as | Concentration (mg $L^{-1}$) | Analyte recovery (%) |
|---|---|---|---|
| $Ca^{2+}$ | $Ca(NO_3)_2$ | 12,500 | 99.8 |
| $Cr^{3+}$ | $Cr(NO_3)_3$ | 100 | 101 |
| $Cu^{2+}$ | $Cu(NO_3)_2$ | 100 | 95.1 |
| $Fe^{3+}$ | $FeCl_3$ | 1000 | 96.2 |
| $K^+$ | $KNO_3$ | 1000 | 101 |
| $Mg^{2+}$ | $Mg(NO_3)_2$ | 100 | 99.4 |
| $Mn^{2+}$ | $MnSO_4$ | 500 | 98.7 |
| $Na^+$ | $NaNO_3$ | 1000 | 99.6 |
| $Zn^{2+}$ | $Zn(NO_3)_2$ | 600 | 97.2 |
| $Cl^-$ | KCl | 500 | 98.6 |
| $I^-$ | KI | 500 | 102 |
| $MoO_4^{2-}$ | $MoO_3$ | 830 | 102 |
| $PO_4^{3-}$ | $NaH_2PO_4$ | 2000 | 99.5 |
| $SeO_3^{2-}$ | $Na_2SeO_3$ | 200 | 101 |

[a] This study was performed using 5 mL of 1 μg $L^{-1}$ Co standard solution.

**Table 4**
Determination of *i*Co in nutritional supplement samples (95% confidence interval; $n = 6$).

| Sample | Added (μg $g^{-1}$) | Found (μg $g^{-1}$) | Recovery (%)[a] |
|---|---|---|---|
| 1 | 0 | [b] | – |
| | 0.50 | 0.51 ± 0.09 | 101 |
| | 1.00 | 0.99 ± 0.06 | 99.1 |
| 2 | 0 | 0.38 ± 0.02 | – |
| | 0.50 | 0.86 ± 0.06 | 97.6 |
| | 1.00 | 1.37 ± 0.11 | 99.5 |
| 3 | 0 | 0.42 ± 0.08 | – |
| | 0.50 | 0.87 ± 0.05 | 95.2 |
| | 1.00 | 1.37 ± 0.10 | 96.5 |
| 4 | 0 | 0.21 ± 0.03 | – |
| | 0.50 | 0.69 ± 0.04 | 97.9 |
| | 1.00 | 1.22 ± 0.12 | 101 |
| 5 | 0 | [b] | – |
| | 0.50 | 0.49 ± 0.07 | 98.6 |
| | 1.00 | 1.02 ± 0.11 | 102 |
| 6 | 0 | [b] | – |
| | 0.50 | 0.49 ± 0.03 | 99.3 |
| | 1.00 | 1.02 ± 0.08 | 102 |

[a] [(Found-base)/added] × 100.
[b] Not detected.

### 3.5. Analytical performance

Extraction efficiencies higher than 90% were obtained when the procedure was developed under optimal experimental conditions (Table 1). The sensitivity enhancement factor ($N_t$), defined as the enrichment factor multiplied by the individual enhancement effects during determination, was also evaluated [28]. The obtained $N_t$ for a sample volume of 5 mL was 60. The relative standard deviation (RSD) resulting from the analysis of 10 replicates of 5 mL solution containing 0.5 μg $L^{-1}$ *i*Co was 4.7%. The calibration graph was linear with a correlation coefficient of 0.9981 at levels near the detection limits and up to at least 3 μg $L^{-1}$ *i*Co. The limit of detection (LOD), calculated based on the signal at intercept and three times the standard deviation about regression of the calibration curve, was 5.4 ng $L^{-1}$ *i*Co for the proposed methodology.

**Table 5**
Performance data obtained by using the proposed method and others IL-based microextraction methods reported for Co determination.

| Method | LOD (ng $L^{-1}$) | RSD (%) | Sample consumption (mL) | Calibration range (μg $L^{-1}$) | Sample | Ref. |
|---|---|---|---|---|---|---|
| CIAME-FO-LADS | 140 | 2.3 | 10 | 1.5–65 | Environmental water | [15] |
| IL-SDME-ETV-ICP-MS | 1.50 | 7.7 | 1.5 | 0.01–50 | Human serum and environmental water | [16] |
| IL-DLLME-ETAAS | 3.80 | 3.4 | 6.0 | 0.038–3.5 | Environmental water, human urine and saliva | [20] |
| IL-SDME-ETAAS | 40.0 | 4.5 | 1.7 | 0.1–2.0 | Environmental water | [18] |
| IL-DLLME-FAAS | 700 | 2.4 | 25 | 2–166 | Environmental water | [19] |
| On Line IL-DLLME-ETAAS | 8.00 | 4.8 | 2.0 | 0.080–7 | Environmental water samples, ophthalmic and parenteral solutions | [14] |
| IL-DLLME-FAAS | 100 | 2.9 | 10 | 0.4–120 | Environmental water, table salt (NaCl) and food grade $NaNO_3$ | [17] |
| USA-TILDLME-ETAAS | 5.40 | 4.7 | 5.0 | 0.050–3 | Nutritional supplements | Present work |

CIAME, cold-induced aggregation microextraction; FO-LADS, fiber optic-linear array detection spectrophotometry; SDME, single-drop microextraction; ETV-ICP-MS, electrothermal vaporization-inductively coupled plasma-mass spectrometry; FAAS, flame atomic absorption spectrometry.

### 3.6. Determination of iCo species in nutritional supplements

The possibility that dietary intake of certain heavy metals for which no function is known could cause imbalances of essential trace minerals in the general population was widely recognized for decades. Specifically about Co, the only evidence of *i*Co being used in food supplements was supplied in the 1960's, when breweries added cobalt sulfate to beer as a foam stabilizer. Several studies reported lethal cardiomyopathy in people who consumed large quantities of beer with cobalt sulfate [29]. Nowadays, there are still no suitable data with which to derive a tolerable intake for chronic ingestion of *i*Co. However, *i*Co (as sulfate) is included in some multiconstituent licensed medicines, at a maximum daily dose of 0.25 mg [30]. On the other hand, *i*Co (sulfate and other soluble Co(II) salts) are possibly carcinogenic to humans (Group 2B) [29]. Therefore, Co species should be monitored in nutrient supplements not only to insurance the nutrient quality of the product (Co as vitamin $B_{12}$) but also in order to avoid potential health problems (as *i*Co). However, as previously mentioned in the introductory section, there is a lack of microextraction methods which are able to discriminate between *i*Co and Co as vitamin $B_{12}$ in nutritional samples.

Therefore, recovery of *i*Co in presence of nutritional supplements matrices was studied in this work. The proposed method was applied to six portions of different matrices and the average concentrations of *i*Co obtained were taken as base values. Due to the non-existence of a certified reference material for *i*Co species in nutritional supplements, the selectivity of the proposed method for *i*Co determination was assayed adding 0.5 and 1 μg $g^{-1}$ *i*Co to samples and the same procedure was followed. The results obtained are summarized in Table 4. *i*Co recoveries were highly satisfactory for all cases independent of matrix composition.

## 4. Conclusions

A highly selective and rapid microextraction method based on $[C_6mim][PF_6]$ IL for selective determination of *i*Co species is presented in this work. The great potential that IL-based microextraction has for trace *i*Co determination, with the help of a selective chelating reagent, such as 1N2N, is demonstrated. Moreover, proper selection of pH is an effective way to selectively extract *i*Co species. This study also indicates that USA-TILDLME can be an excellent and green extraction technique for *i*Co separation and preconcentration, even from complex matrices like nutritional supplement ones, showing good tolerance to possible interferences caused by other co-existing metal ions.

All in all, the results indicate that the proposed procedure is a simple, fast, interference-free, selective and environment-friendly analytical approach. Our method involving TILDLME technique, improved by ultrasound and combined with ETAAS detection, presents a limit of detection comparable to, or better than other methodologies based on ILs for Co extraction (Table 5). Likewise, most of the IL-based methodologies previously proposed for Co extraction were applied to samples with low complex matrices such as environmental waters. On the other hand, the proposed preconcentration method was successfully applied for *i*Co determination in highly complex samples such as nutritional supplements, with good accuracy and good reproducibility. Finally, the proposed method shows good calibration range with a reduced amount of sample, with the additional advantage of using low cost and widely spread instrumentation.

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