www.elsevier.com/locate/talanta

Development of an ionic liquid-based dispersive liquid-liquid microextraction method for the determination of nifurtimox and benznidazole in human plasma

J.M. Padró, M.E. Marsón, G.E. Mastrantonio, J. Altcheh, F. García-Bournissen, M. Reta

PII: S0039-9140(13)00002-7
DOI: http://dx.doi.org/10.1016/j.talanta.2012.12.050
Reference: TAL13558

To appear in: *Talanta*

Received date: 4 December 2012
Revised date: 10 December 2012
Accepted date: 13 December 2012

Cite this article as: J.M. Padró, M.E. Marsón, G.E. Mastrantonio, J. Altcheh, F. García-Bournissen and M. Reta, Development of an ionic liquid-based dispersive liquid-liquid microextraction method for the determination of nifurtimox and benznidazole in human plasma, *Talanta,* http://dx.doi.org/10.1016/j.talanta.2012.12.050

DEVELOPMENT OF AN IONIC LIQUID-BASED DISPERSIVE LIQUID-LIQUID MICROEXTRACTION METHOD FOR THE DETERMINATION OF NIFURTIMOX AND BENZNIDAZOLE IN HUMAN PLASMA

Padró, J. M[a], Marsón, M. E.[b], Mastrantonio, G. E.[b,c], Altcheh, J.[d], García-Bournissen, F.[d], Reta, M.*[,a]

[a]*Laboratorio de Separaciones Analíticas, División Química Analítica, Facultad de Ciencias Exactas, UNLP, ARGENTINA.*

[b]*Área de Toxicología, Facultad de Ciencias Exactas, UNLP, ARGENTINA.*

[c]*Laboratorio de Servicios a la Industria y al Sistema Científico (LaSeISiC). UNLP / CIC-PBA, La Plata, ARGENTINA.*

[d] *Parasitología-Chagas. Hospital de Niños Ricardo Gutiérrez, Buenos Aires, ARGENTINA.*

Abstract

Dispersive ionic liquid-liquid microextraction combined with liquid chromatography and UV detection was used for the determination of two antichagasic drugs in human plasma: nifurtimox and benznidazole. The effects of experimental parameters on extraction efficiency—the type and volume of ionic liquid and disperser solvent, pH, nature and concentration of salt, and the time for centrifugation and extraction—were investigated and optimized. Matrix effects were detected and thus the standard addition method was used for quantification. This microextraction procedure yielded significant improvements over those previously reported in the literature and has several advantages, including high inter-day reproducibility (relative standard deviation = 1.02% and 3.66% for nifurtimox and benznidazole, respectively), extremely low detection limits (15.7 ng mL^{-1} and 26.5 ng mL^{-1} for nifurtimox and benznidazole, respectively), and minimal amounts of sample and extraction solvent required. Recoveries were high (98.0% and 79.8% for nifurtimox and benznidazole, respectively). The proposed methodology offers the advantage of highly satisfactory performance in addition to being inexpensive, simple, and fast in the extraction and preconcentration of these antichagasic drugs from human-plasma samples, with these characteristics being

consistent with the practicability requirements in current clinical research or within the context of therapeutic monitoring.

Highlights

- An analytical procedure for the determination of two antichagasic drugs in plasma was proposed.
- The procedure yielded a significant improvement over those reported in the literature.
- The performance of the methodology was very satisfactory and requires very low amount of sample.
- The technique is according to requirements in clinical research or therapeutic monitoring.

Keywords: Benznidazole; Nifurtimox; Antichagasic drugs; Dispersive liquid-liquid microextraction; Liquid chromatography; Ionic liquids

Introduction

Chagas disease, also known as American trypanosomiasis—first described by Carlos Chagas in 1909 [1]—is a potentially life-threatening illness caused by the protozoan parasite *Trypanosoma cruzi (T. cruzi)*. Chagas occurs mainly in Latin America, where transmission to humans is effected either through the feces of triatomine bugs or, in some cases, congenitally [2]. The disease affects approximately 16 to 18 million people, and more than 100 million people are exposed to the risk of infection [3]. In 2008 over 10,000 people were estimated to have died of Chagas disease. Because of the nonvector routes of infection—such as from mother to child, through blood transfusion, or via organ transplantation—the transmission of *T. cruzi* and the disease itself are no longer limited to Latin America, but rather have now become a worldwide problem [4,5]. Chagas disease has been rising in the ranking of international health priorities as a result of the growing extent of migration from endemic to nonendemic areas such as North America and Europe [6,7].

Benznidazole (*N*-benzil-2-nitroimidazolylacetamide, BNZ) and nifurtimox (3-methyl-*N*-[(5-nitro-2-furanyl)-methylene]-4-thiomorpholinamine-1,1-dioxide, NFX) are the only two drugs currently available for the treatment of Chagas disease, although BNZ is available in all the affected countries [8]. Both medicines are almost 100% effective in curing the disease if given soon enough after infection—*i. e.*, up to the onset of

the acute phase. The efficacy of both drugs, however, diminishes the longer a person has been infected [1]. Nevertheless, the pharmacologic treatment of adults is associated with a greater than 30% incidence of adverse drug reactions [9], especially neuropathy and severe dermatologic and gastrointestinal symptoms, leading to treatment interruption in over 20% of the patients [10,11]. These pharmacologic characteristics imply the need for a close monitoring of the therapeutic agents.

BNZ is a chemotherapeutic drug currently used for the treatment of *T. cruzi* infections in both the chronic and acute phases. A few reports have been published on detection methods for BNZ. Raaflaub and Ziegler [12] investigated the bioavailability of the compound in plasma using polarography. Walton and Workman [13] determined BNZ and its metabolized amine derivative in blood plasma by high-performance liquid chromatography with ultraviolet detection (HPLC-UV) at a recovery of 90% and a reproducibility of 3.2%. Barbeira and coworkers have studied direct–current and differential-pulse–polarographic methods for the analysis of BNZ in pharmaceutical formulations [14]. La-Scalea *et al.* investigated the voltametric behavior of BNZ with a glassy-carbon electrode and a DNA-biosensor [15]. The latter enabled the study of BNZ–DNA interactions through the use of immobilized DNA on the glassy–carbon–electrode surface. Only a few authors have developed an HPLC method to quantify BNZ in plasma and/or urine for further implementation in human pharmacokinetic and health-safety studies [12,16-18].

A few methods have been published for the determination of' NFX in biologic fluids, including colorimetry with thin-layer chromatography [19] and HPLC [16,20]. By the former methodology, the results obtained of assays in serum, plasma, and urine after the oral administration of NFX to rats, dogs, and humans permitted a quantitative determination of the drug at a sensitivity of at least 0.5 μg mL^{-1}. The latter approach resulted in the development of an easy sample-preparation procedure for pharmacokinetic studies in patients with chronic renal failure [19].

The determination of clinically significant plasma BNZ or NFX concentrations has generated considerable interest. To our knowledge, two different contexts exist: the plasma concentrations of adults and children. A therapeutic range between 3 and 6 μg mL^{-1} in adult-plasma samples was originally proposed for both drugs on the basis of *in-vitro* data and the results from pharmacokinetic studies in adult humans, but lower values were observed for BNZ in the pediatric patients [21]. Similar values were obtained for NFX [20,22]. Recently, J. Altcheh and colleagues reported a high efficacy of BNZ in pediatric Chagas disease

84 despite the use of lower plasma concentrations than had been reported in adults. Thus, the plasma
85 concentration of antichagasic drugs at the lower limit of clinical significance has yet to be determined
86 definitively, especially in pediatric pharmacotherapeutics [21].

87 Dispersive liquid-liquid microextraction (DLLME)—a novel method recently developed by Assadi
88 and coworkers [23,24]—has been applied for the determination of several analytes in different matrices. This
89 method is based on a ternary solvent system in which the extraction solvent (*e. g.*, dichloromethane, octanol,
90 toluene) and the miscible disperser solvent (*e. g.*, methanol, acetonitrile, isopropanol) are rapidly injected into
91 the aqueous sample by a syringe. The disperser solvent must be miscible with both the aqueous and the
92 organic phases. At the beginning of the dispersion, exceedingly small droplets (with therefore a major
93 surface-contact area) are formed that enable a maximal increase in mass transfer. Those droplets then collapse
94 to form the ionic-liquid phase containing the analytes in an extremely small volume, thus achieving high
95 enrichment factors. This last step can be speeded-up by centrifugation.

96 Room-temperature ionic liquids (RTILs)—a form of melting salts composed of organic cations and
97 either organic or inorganic anions—have emerged as possible environmentally friendly solvents (aka *green*
98 solvents) [25,26] and thus have achieved a wide application in the separation sciences [27-29], among other
99 research areas, because of their unique properties—namely: low volatility, chemical and thermal stability, and
100 good solubility in both organic and inorganic solvents. RTILs are progressively replacing the typical organic
101 solvents in sample preparations. Ionic liquids (ILs) have been used as extractants in DLLME (*i. e.*, for IL-
102 DLLME) in several studies such as the determination of nonsteroidal anti-inflammatory drugs in urine by
103 liquid chromatography and the ultraviolet detection [30] of insecticides [31] or polyaromatic hydrocarbons
104 [32] in water samples.

105 In this investigation, we applied the IL-DLLME technique combined with HPLC-UV for the first
106 time for the determination of BNZ and NFX levels in human plasma and both determined and optimized the
107 effect of the critical experimental parameters on the extraction efficiency—namely, the nature and volume of
108 the IL and disperser solvent, the pH, the type and concentration of salt, and the extraction and centrifugation
109 times.

110

111 **2. Experimental**

112 **2.1. Chemicals and materials**

113 1-hexyl-3-methylimidazolium hexafluorphosphate, ([HMIM][PF_6], ≥97.0% purity) was purchased
114 from Fluka, Buchs, Germany. 1-butyl-3-methylimidazolium hexafluorphosphate ([BMIM][PF_6]), 1-octyl-3-
115 methylimidazolium tetrafluoroborate([OMIM][BF_4]), and 1-octyl-3-methylimidazolium hexafluorphosphate
116 ([OMIM][PF_6]), were synthesized in our laboratory through an adaptation of a procedure from the literature
117 [29]. Reagents were of analytical grade or better: benznidazole (Roche, Buenos Aires, Industria Argentina),
118 nifurtimox (Bayer, Leverkusen, Germany), 1-bromobutane, 98.0% (Riedel-de-Haën, Seelze, Germany),
119 potassium hexafluorphosphate, 98.0% (Aldrich, WI, USA), 1-methylimidazole, ≥99.0% and phosphoric acid,
120 85% w/w (Merck, Hohenbrunn, Germany), tetrafluoroboric acid, 48.0% w/v in water (Sigma-Aldrich, St.
121 Louis, MO, USA), 1-bromoctane, 99.0% (Aldrich, WI, USA), hydrochloric acid and acetone, (Merck, Buenos
122 Aires, Argentina), sodium hydroxide (Analar, Poole, England), potassium chloride, sodium chloride,
123 trichloroacetic acid, sodium phosphate dibasic anhydrous and sodium bicarbonate (Anedra, Argentina),
124 potassium phosphate (Matheson, Coleman & Bell, Norwood, OH, USA), magnesium sulfate 7-hydrate
125 (Biopack, Argentina), potassium phthalate monobasic, ≥99.5% (Fluka, Buchs, Germany), sodium borate and
126 methanol HPLC grade (Baker´s Analyzed, Phillipsburg, NJ, USA), acetonitrile and anhydrous ethanol (Carlo
127 Erba, Divisione Chimica Industriale–Milano, Italy). Solutions were prepared with MilliQ® water.

128 The 100-µL and 25-µL microsyringes were respectively supplied by Hamilton, Reno, NV, USA and
129 Agilent Technologies, Australia. The micropipettes were purchased from Eppendorf, Hamburg, Germany.

130 Conical graduated polypropylene light-blue screw-capped test tubes (17 x 120 mm, 15 mL) were used
131 and the samples filtered through a Micro-Mate™ interchangeable syringe (Popper & Sons Inc., New Hyde
132 Park, NY) containing a 0.22-µm cellulose-nitrate membrane.

133

134 **2.2. Instrumentation and chromatographic condition**

135 An HP 1100 liquid chromatograph equipped with a binary pump, a thermostat-controlled column
136 compartment, degasser, and variable-wavelength detector connected to a Data Apex CSW workstation (Data
137 Apex, Czech Republic) was used. Chromatographic analysis was performed on a 250x4.6-mm ID (5-µm)
138 Zorbax Eclipse XDB-C18 column (Agilent). Methanol-containing buffered phosphate (58:42; pH 2.70, 25
139 mM) was used in the mobile phase. The organic phase was prefiltered through a 0.22-µm nylon membrane

(Osmonics-Magna) and the aqueous phase was prefiltered through a 0.45-μm cellulose-nitrate membrane (Micron Separations). The detector was set at 320 nm for BNZ and 395 nm for NFX, at which wavelengths the RTILs studied absorb no radiation. The injector (Rheodyne Model 7725i, Cotati, CA, USA) was fitted with a 5-μL loop. The flow rate was set at 1 mL min^{-1}.

A LUGUIMAC LC-20 centrifuge operating at 4,200 rpm with 15-mL polypropylene tubes were used for the optimization experiments and an Eppendorf 5417 C/R centrifuge operating at 4,200 rpm for the quantification experiments—and the latter because of the low amounts of sample available. A Vortex Genie 2 (Scientific Industries, Inc., USA) mixer was used for mixing the aqueous and the IL phases, and a combined glass Metrohm electrode in a commercial Accument AR 25 pH/mV/Ion/Meter (Fisher Scientific) pH meter gave the pH measurements. Water was purified with a Milli-Q system (Millipore Co.).

2.3. Extraction procedure for the optimization experiments

The IL-DLLME was performed according to the following optimized procedure (see Section 3): 5.00 mL of aqueous solution spiked with NFX (9.4 μg mL^{-1}) or BNZ (5.6 μg mL^{-1}) was placed in a 15-mL conical centrifuge tube. A mixture of 125 μL [OMIM][PF_6] saturated with water and 0.30 mL methanol (the disperser solvent) was injected into the sample solution with a micropipette. After vortex-mixing, a cloudy solution was quickly formed. To increase the extraction efficiency, 0.1 g of KCl had been added. The analytes in the aqueous sample had become extracted into the fine ionic-liquid droplets at this step, while the methanol remained miscible in the aqueous solution. The mixture was then shaken for 6 min and centrifuged at 4,200 rpm for 20.0 min. After this centrifuging, the droplets of ionic liquid had completely collected at the bottom of the centrifuge tube. The upper, aqueous phase was removed with a Pasteur pipette without disturbing the underlayer. The IL-phase volume was 120 ± 5 μL. Of the sedimented phase, 5 μL was withdrawn and injected into the HPLC column. All experiments were performed in triplicate.

2.4. Extraction procedure for the human-plasma samples

Human plasma spiked with different amounts of NFX and BNZ were acidified with 30% (w/v) trichloroacetic acid solution (0.10 mL in 1.00 mL of the sample) to remove the proteins. The mixture was then

shaken in a vortex for 6 min and centrifuged for 20 min. The supernatant was decanted and filtered through 0.22-μm membranes into a 15-mL polypropylene tube.

The IL-DLLME was performed according to the following microscale-adapted procedure from Section 2.3: (1) To 540 μL of the supernatant, placed in a 2.0-mL polypropylene microcentrifuge tube, was added 32.4 μL of methanol containing 13.5 μL of [OMIM][PF_6]. A cloudy solution resulted immediately as the analytes in the water sample became extracted into the fine droplets of the ionic liquid that were formed. (2) After adding 30 μL 1M NaOH to adjust the pH to the optimum (pH=6.12) along with 0.011g of KCl, the salting-out effect was produced (3) The cloudy solution was vortex-mixed for 6 min, then centrifuged for 20.0 min at 4,200 rpm to sediment the previously dispersed fine droplets of the ionic liquid into a unified volume at the bottom of the microcentrifuge tube. (4) Of this sedimented phase, 5 μL were withdrawn with a 25-μL microsyringe and then injected into the HPLC system for quantification.

2.5. Preparation of stock and standard solutions in water and the sample matrix

Stock solutions of NFX and BNZ were prepared by dissolving the compounds in methanol at concentrations of 940 and 560 μg mL^{-1}, respectively. The solutions were sonicated for a few minutes in order to accelerate the dissolution. These stocks were stored in the refrigerator for up to one month and their preservation status checked daily by comparing the areas of relevant chromatographic peaks with the corresponding values obtained immediately after the solutions were prepared.

The standard solutions for the calibration curves were prepared both in water and in human plasma to evaluate possible matrix effects. Calibration curves in water were prepared by diluting the stock solutions with MilliQ® water and filtering through 0.22-μm cellulose-nitrate membranes.

Calibration curves in the sample matrix were prepared from human plasma free of NFX and BNZ. The samples were spiked with different volumes of the standard solutions. The solutions thus obtained were extracted by the procedure described in Section 2.5. Without dilution with any organic solvent, 5 μL of the resulting sedimented RTIL was injected into the HPLC column and analyzed under the aforementioned chromatographic conditions. The curves were obtained by plotting the peak areas *vs*. the concentrations of the analytes in the human plasma.

3. Results and discussion

3. 1. Optimization of IL-DLLME

In order to choose the best experimental extraction conditions, a constant volume (5.00 mL) of the standard solution (*cf.* Section 2.6) was used in all the optimization experiments. As a consequence, in these experiments we did not use a specific concentration and, thus, the results shown in the figures correspond to the chromatographic areas reflecting the amount of analyte extracted into the IL phase relative to a constant initial amount. In the experimental procedure, a step-by-step optimization scheme was designed. Some significant parameters that would affect the extraction performance—namely, the nature and volume of the extraction and disperser solvents, the extraction and centrifugation times, the pH of the aqueous samples, and the type and salt concentration (for the salting-out effect) were studied and optimized.

3. 1. 1. Selection of the ionic liquid

To select a given ionic liquid for a particular extraction is quite difficult since several water-immiscible room-temperature ILs are commercially available [33-35]. The IL of choice should have a low miscibility in water, be denser than the matrix solution so that the microdroplets can be cleanly sedimented in order to be able to completely discard the aqueous phase thereafter, have good chromatographic behavior and a strong extraction affinity for the compound of interest, be inexpensive, and finally be directly injectable into the HPLC column. This last requirement, however, is not usually met since the IL must have a high viscosity, thus needing the addition of an organic solvent to make the organic phase sufficiently fluid before injection; and this step decreases the enrichment factor. For all these reasons, we selected the following imidazolium-based ILs containing hexafluorophosphate or tetrafluoroborate anions with different alkyl chains: [BMIM][PF_6], [HMIM][PF_6], [OMIM][PF_6] and [OMIM][BF_4].

Fig. 2 compares the extraction performance for the four ILs. The extractions were made in triplicate with the same initial volume of the standard solution (5.00 mL) and the same volume of the methanol-IL mixture (*i. e.*, 0.50 mL/40 μL). All tubes were centrifuged for 20.0 min at 4,200 rpm. The IL [OMIM][PF_6] produced the best extraction performance for both antichagasic drugs probably because stronger hydrophobic interactions were established between the longer alkyl chain of the IL and the analytes (Fig. 2). Thus, that IL was used for all of the subsequent experiments.

223

224 ***3. 1. 2. Selection of disperser solvent***

225 The key feature of consideration for the selection of disperser solvent is the miscibility in both the IL
226 phase (the extraction solvent) and the aqueous sample. Acetone, ethanol, acetonitrile, and methanol were
227 considered in this experiment. A series of sample solutions were studied containing 0.50 mL of each disperser
228 solvent plus 40 µL of [OMIM][PF_6]. Since the chromatographic areas and, as a consequence, the extraction
229 yields for the two drugs were found to be higher when methanol was used as the disperser solvent (Fig. 3),
230 methanol was chosen for the subsequent experiments.

231

232 ***3. 1. 3. Amount of ionic liquid***

233 To evaluate the effect of the amount of IL, a constant volume of methanol (0.50 mL) containing
234 different volumes of [OMIM][PF_6] were used. By increasing the amount of IL, the extraction efficiency
235 increased for the two antichagasic drugs, but after a maximum volume of 125 µL the chromatographic areas
236 were seen to decrease (Fig, 4). Consequently, 125 µL of the IL was used as the optimum quantity for the
237 sample extractions.

238

239 ***3. 1. 4. Amount of disperser solvent***

240 The volume of disperser solvent affects the solubility of the extraction solvent in the aqueous solution
241 and, thus, the volume of sedimented phase. To obtain the optimal volume, experiments were performed with
242 different methanol volumes containing the optimized amount of IL. The extraction was seen to increase up to
243 0.30 mL of methanol as the result of a better solubilization of the IL which liquid therefore became atomized
244 into progressively smaller microdroplets (Fig. 5). By increasing the volume of methanol, however, the
245 extraction yield decreased because of a greater partitioning of the analytes into the aqueous phase. Thus, 0.30
246 mL of methanol was indicated as the optimum volume.

247

248 ***3. 1. 5. Effect of pH***

249 The effect of pH on the extraction efficiency was carried out within the pH range of 2.39 to 10.03.
250 Different buffers were used depending on the desired pH (potassium phthalate monobasic at pH = 2.39 and

4.52, sodium phosphate at pH = 6.12, sodium borate at pH = 8.44 and sodium bicarbonate at pH = 10.03), but the ionic strength was kept constant (0.1 M) throughout. A mixture of 0.30 mL of methanol and 125 μL [OMIM][PF_6], was quickly added to the sample solution (Fig. 6). Although, to the best of our knowledge, the relevant pK_a values were not available in the literature, the extraction proved to be maximum at pH = 6.12 so this pH was chosen for the experiments (Fig. 6).

3. 1. 6. Salt effects

The effect of salt addition was determined with four different salts: NaCl, KCl, $MgSO_4$, and K_3PO_4. The extraction efficiency depended on the type and concentration of the salt added (Fig. 7). Previous reports had indicated that when certain salts were used in the aqueous phase for extraction with ILs, the electrostatic interaction between the salt ions and the IL ions enhanced the solubility of the IL in the aqueous phase and thus undermined the extraction efficiency [36-38]. In the present work, this effect was observed for NaCl, but for the other three salts an initial increase in the extraction was obtained as a result of the well known salting-out effect. Furthermore, when KCl was used, the amount of recovered analyte was much higher than with the other salts (*cf.* the *y*-axes in Figs. 7A-D). Since for this salt a maximum was reached at 2.00% (w/v), this concentration of KCl was used.

3. 1. 7. Extraction and centrifugation times

The centrifugation time was defined as the length of time the tube was inside the centrifuge. Centrifugation helps to separate the IL phase from the aqueous phase particularly when highly viscous ILs tend to stick to the microtube wall, as occurred in this study. A series of extractions was performed with the centrifugation times varied from 3.0 to 60.0 min at 4,200 rpm, the maximum speed of the centrifuge. Since the chromatographic-peak area plateaued at 20 min (Fig. 8.A), this centrifugation time was considered optimal.

The extraction time was defined as the interval between the instant when the IL was added to the sample solution through the time in which both phases were in contact during shaking. This extraction time was varied between 1 and 20 min. The extraction efficiency increased up to 6.0 min and then reached a

plateau (Fig. 8.B). The two solvents obviously required a minimum time to reach equilibrium with the analyte and then separate.

3.2. Analytical performance of the proposed methodology

The IL-DLLME–HPLC-UV method as developed in this systematic manner was then applied to the determination of BNZ and NFX in human plasma. The following figures of merit were evaluated: accuracy, reproducibility, limit of detection (LOD), limit of quantification (LOQ), the linearity range (LR), enrichment factor (EF), and extraction recovery (R%).

Calibration curves (Table 1) were made by linear regression of the peak areas *vs.* concentration in both water (thirteen levels) and human plasma (eight levels) for NFX and for BNZ. All determinations were made in triplicate.

In order to investigate if matrix effects were present for the quantitative determinations, we compared the slopes of the calibration curves obtained by the external-standard method for analytes dissolved in water with the slopes obtained by spiking the plasma samples. For the purpose of these comparisons, we chose the *t*-test according to Equation 1 [39]:

$$t = \frac{b_1 - b_2}{\sqrt{s_{b_1}^2 + s_{b_2}^2}} \qquad (1)$$

where b_1 and b_2 are the slopes of the regression equations to be compared and s_{b1} and s_{b2} are the respective standard deviations. If the residual variances s_e^2 for both sets of data are equal (according to an *F*-test), a so-called *combined standard deviation* can be calculated to obtain a *t*-value for comparison with tabulated values for n_1+n_2-4 degrees of freedom. If, however, the residual variances are not equal, the Cochran test for the comparison of two slopes with unequal variances must be used. Thus, if s_{b1}^2 is different of s_{b2}^2, then theoretical *t*-values, t_1 and t_2, at the chosen level of significance and n_1-2 and n_2-2 degrees of freedom, respectively, can be obtained from a *t* table. Next, a *combined t′ value* can be calculated by Equation 2, which figure can finally be compared with the calculated *t* values through the use of equation 1:

$$t' = \frac{t_1 s_{b_1}^2 + t_1 s_{b_2}^2}{s_{b_1}^2 + s_{b_2}^2} \qquad (2)$$

The calculation of t' is not necessary if both regression lines are based on the same number of data points ($n_1 = n_2$), in which circumstance $t' = t_1 = t_2$. The working curve in human plasma was compared with the standard one in water to detect matrix effects. For BNZ, the t' value was 2.045, and the t was 3.07; whereas for NFX, the t' value was 2.025, and the t was 58.95. Thus, since the t was higher than the t' in both instances, we could conclude that the slopes were significantly different so that matrix effects were therefore present. As a consequence, for the quantification of BNZ and NFX in plasma samples, the standard addition method was used.

The LOD in human plasma was calculated by different procedures in order to make comparisons with other studies in the literature—for example, by using the signal to noise ratio (S/N) = 2.0, 3.0 and by using the IUPAC definition of LOD = 3.29 s_o [39] (based on the standard deflection of the concentration predicted for a blank sample, s_0).

The lower LOQ in human plasma (at the beginning of the linear range) was evaluated by the S/N of 10, and by the IUPAC definition of LOQ at 10 s_0 [39]. The end point of the linear range (*i. e.*, the upper limit of quantification) was determined by the lack-of-fit procedure [39]—*i. e.*, by eliminating the highest value and applying the statistical test again with the remaining points. This process is repeated until the data can be adjusted to a straight line.

In order to validate the accuracy and precision of the determinations, each sample was spiked with the target drug at three different concentrations within the linear range of the calibration curve. The precision of the NFX and BNZ assay was determined by the repeatability (intra-day) and reproducibility (inter-day determinations) with samples containing 5.0, 7.5, and 10.0 μg mL^{-1} of NFX and BNZ. Reproducibility was expressed as the percent relative standard deviation (%RSD) with respect to measurements made in triplicate. The same drug concentrations were analyzed over three consecutive days to determine inter-day precision.

The enrichment factors (EFs) and recoveries (%Rs) were calculated by means of equations 3 and 4, respectively:

$$EF = \frac{C_{IL}}{C_{aq}} \qquad (3)$$

where C_{IL} and C_{aq} are the analyte concentrations in the IL phase and the initial aqueous solution, respectively,

$$R\% = 100\frac{C_{IL}V_{IL}}{C_{aq}V_{aq}} = 100.EF.\varphi \quad (4)$$

where V_{IL} and V_{aq} are the volumes of the IL phase and the sample solution, respectively, and φ is the phase ratio. Plasma human samples were spiked with known volumes of NFX and BNZ solutions of known concentration (2.5, 5.0, 7.5, and 10.0 μg mL^{-1}). The concentrations of the target analytes in the extracts were within the linear range of the calibration curves. The EF was calculated by Equation 3, after spiking a measured amount of analyte in a known volume of plasma and then determining the final concentration extracted into the RTIL phase. The analyte was left in contact with the plasma matrix for one hour before extraction. Recovery was determined by measuring the initial volume of spiked plasma and the final volume of RTIL phase and using the EF obtained in Equation 4. The recoveries obtained for NFX at different concentration levels were higher than those for BNZ (Table 3). As was discussed in our previous paper, the hydrophobic-interaction determines the extraction process with RTILs [29 and references therein]. Fig. 9 shows the typical chromatograms of the two antichagasic drugs before and after IL-DLLME, in a spiked human plasma sample. The original sample was clearly spiked at a level in which the subsequent chromatographic-peak area could be measured (where the drug concentration for a sample obtained from a treated patient would not necessarily be so propitious). Table 4, however, further provides the antichagasic-drug contents of human-plasma samples determined after the IL-DLLME was spiked to give a range of different concentrations of those agents. Nevertheless, even in this circumstance, the precision and reproducibility obtained for each level remained satisfactorily high.

The methodology for the determination of BNZ and NFX in human plasma proposed here was then compared with other methods extant in the literature, mostly consisting of direct matrix analyses by HPLC with UV detection or thin-layer chromatography. The LOD, LOQ, LR, reproducibility (%RSD), amount of sample necessary for the analysis, type and amount of solvent, and R% are presented in Table 5. Compared to these earlier examples, the IL-DLLME–HPLC-UV technique requires small amounts of extraction solvent (here a few microliters of an ionic liquid) and exhibits a wide range of linearity, very low limits of detection and quantification, and excellent reproducibility within and between samplings. The recoveries were moreover high for both NFX and BNZ.

The methodology developed and described here is now being applied to the study of real samples—*i. e.*, human plasma from infected patients—for future presentation of the findings.

3.3. Conclusions

The IL-DLLME–HPLC-UV technique has been used here for the first time to analyze BNZ and NFX in human-plasma samples. The experimental conditions for the extraction of these analytes have been investigated and optimized. Although a step-by-step procedure to obtain the optimum extraction conditions was used, the analytical methodology proved to have several advantages compared to other previously reported extraction techniques—namely, better reproducibility, lower detection limits, and the requirement for much lower amounts of extraction solvent. Moreover, the technique requires very small amounts of sample, which characteristic in the example of human plasma is a highly practical and desirable feature. Recovery was notably high for both compounds, and the performance of the proposed methodology was most satisfactory. Thus, the IL-DLLME-HPLC-UV technique promises to be a simple, fast, efficient, and facile method for the enrichment and quantitative determination of BNZ and NFX in human-plasma samples.

3.4. Acknowledgements

Financial support from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Técnica (ANPCYT), Fundación Bunge & Born, Programa Salud Investiga (Ministerio de Salud de la República Argentina), Facultad de Ciencias Exactas (UNLP), and Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC-PBA) is gratefully acknowledged.

M. Reta is a scientific member of CONICET and Professor of Analytical Chemistry at the Universidad Nacional de La Plata (Argentina).

The authors also wish to thank Dr. Donald F. Haggerty, a retired career investigator and native English speaker, for editing the final version of the manuscript.

3.5. References

[1] Chagas Disease (American trypanosomiasis), Fact Sheet N° 340 (2010)

World Health Organization; http://www.who.int/mediacentre/factsheets/fs340/en/index.html.

387 [2] F. García-Bournissen, J. Altcheh, C. O. Della Védova, N. Giglio, G. Mastrantonio, G. Koren, Paediatr.
388 Drugs 11 (2009) 33-37.
389 [3] S. Garcia, C. O. Ramos, J. F. V. Senra, F. Vilas-Boas, M. M. Rodrigues, A. C. Campos-de-Carvalho, R.
390 Ribeiro-dos-Santos, M. B. P. Soares, Antimicrob. Agents Chemother. 49 (2005) 1521-1528.
391 [4] J. Gascon, C. Bern, M. J. Pinazo, Acta Trop. 115 (2010) 22-27.
392 [5] M. Piron, M. Verges, J. Muñoz, N. Casamitjana, S. Sanz, R. M. Maymo, J. M. Hernandez, L. Puig, M.
393 Portus, J. Gascon, S. Sauleda, Transfusion 48 (2008) 1862-1868.
394 [6] G. A. Schmunis, Mem. Inst. Oswaldo Cruz 102 (2007) 75-85.
395 [7] C. Di Girolamo, C. Bodini, B. L. Marta, C. Ciannameo, F. Cacciatore Eurosurveill. 16 (2011) 5.
396 [8] L. Guerrero, M. J. Pinazo, E. Posada, J. Gascón, J. Ribas, D. Soy, Clin. Chem. Lab. Med. 49 (2011) 77-82.
397 [9] J. A. Castro, M. Montalto de Mecca, L. C. Bartel, Hum. Exp. Toxicol. 25 (2006) 471-479.
398 [10] R. Viotti, C. Vigliano, B. Lococo, M. G. Alvarez, M. Petti, G. Bertocchi, A. Armenti, Expert. Rev. Anti.
399 Infect. Ther. 7 (2009) 157-163.
400 [11] J. Altcheh, G. Moscatelli, S. Moroni, F. García-Bournissen, H. Freilij, Pediatrics 127 (2011) 212-218.
401 [12] J. Raaflaub, W. H. Ziegler, Arzneim. Forsch 29 (1979) 1611-1614.
402 [13] M. I. Walton, P. Workman, J. Chromatogr. B 375 (1986) 190-196.
403 [14] P. J. S. Barbeira, G. M. Silva, M. D. L. P. M. Beatriz, N. R. Stradiotto, J. Pharm. Biomed. Anal. 20
404 (1999) 723-726.
405 [15] M. A. La-Scalea, S. H. P. Serrano, E. I. Ferreira, A. M. Oliveira Brett, J. Pharm. Biomed. Anal. 29
406 (2002) 561-568.
407 [16] R. F. Bulffer, J. A. Castro, S. L. Fanelli, Acta Bioquim. Clin. Latinoam 45 (2011) 463-470.
408 [17] L. C. Bartel, M, Montalto de Mecca, C. Rodríguez de Castro, F. M. Bietto, J. A. Castro, Human Exp.
409 Toxicol., 29 (2010) 813-822.
410 [18] R. Moreira da Silva, L. Teixeira Oliveira, N.M. Silva Barcellos, J. de Souza, M. de Lana,
411 Antimicrob.Agents Chemother. 56 (2012) 3344-3348)
412 [19] H. Medenwald, K. Brandau, K. Schlossmann, Arzneim. Forsch 9 (1972) 1613-1617.
413 [20] C. Paulos, J. Paredes, I. Vasquez, G. Kunze, G. González-Martín, J. Chromatogr. B 433(1988) 359-362.
414 [21] J. Altcheh, G. Moscatelli, G. Mastrantonio, S. Moroni, N. Giglio, G. Koren, H. Freilij, F. García-

415 Bournissen (2010) 16th World Congress of Basic and Clinical Pharmacology, 17-23 July 2010, Copenhagen,
416 Denmark. Basic Clin. Pharma. Toxic. 107 (2010) (Suppl.1)162-692.
417 [22] G. González-Martin, S. Thambo, C. Paulos, I. Vasquez, J. Paredes, Eur. J. Clin. Pharmacol. 42 (1992)
418 671-673.
419 [23] M. Rezaee, Y. Assadi, M. R. Milani Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, J. Chromatogr. A
420 1116 (2006) 1-9.
421 [24] S. Berijani, Y. Assadi, M. Anbia, M. R. Milani Hosseini, E. Aghaee, J. Chromatogr. A 1123 (2006) 1-9.
422 [25] N. M. M. Mateus, L. C. Branco, N. M. T. Lourenco, C. A. M. Afonso, Green Chem. 5 (2003) 347-352.
423 [26] H. Z. Yang, Y. L. Gu, Y. Q. Deng, F. Shi, Chem. Commun. 3 (2002) 274-275.
424 [27] D. Qi, S. Y. Cui, X. G. Chen, Z. D. Hu, J. Chromatogr. A 1059 (2004) 191-198.
425 [28] J. G. Huddleston, H. D. Willauer, R. P. Swatloski, A. E. Visser, R. D. Rogers, Chem. Commun. 16
426 (1998) 1765-1766.
427 [29] J. M. Padró, A. Ponzinibbio, L. B. Agudelo Mesa, M. Reta, Anal. Bioanal. Chem. 399 (2011) 2807-2820.
428 [30] M. Cruz-Vera, R. Lucena, S. Cárdenas, M. Valcárcel, J. Chromatogr. A 1216 (2009) 6459–6465.
429 [31] Y. Liu, E. Zhao, W. Zhu, H. Gao, Z. Zhou, J. Chromatogr. A 1216 (2009) 885–891.
430 [32] M. T. Pena, M. C. Casais, M. C. Mejuto, R. Cela, J. Chromatogr. A 1216 (2009) 6356–6364.
431 [33] X. H. Zang, Q. H. Wu, M. Y. Zhang, G. H. Xi, Z. Wang Chin. J. Anal. Chem. 37 (2009)161-168.
432 [34] R. R. Kozani, Y. Assadi, F. Shemirani, M. R. Milani Hosseinni, M. R. Jamali, Talanta 72 (2007) 387-
433 393.
434 [35] J. G. Huddleston, A. E. Visser, W. M. Reichert, H. D. Willauer, G. A. Broker, R. D. Rogers, Green
435 Chem. 3 (2001) 156-164.
436 [36] J. F. Peng, J. F. Liu, X. L. Hu, G. B. Jiang, J. Chromatogr. A 1139 (2007) 165-170.
437 [37] L. Y. Fu, X. J. Liu, J. Hu, X. L. Zhao, H. L. Wang, Anal. Chim. Acta 632 (2009) 289-295.
438 [38] Q. B. H. Zhou, G. Xie, J. Xiao, J. Chromatogr. A 1177 (2008) 43-49.
439 [39] B. G. M. Vandeginste, D. L. Massart, L. M. C. Buydens, S. De Jong, P. J. Lewi, J. Smeyers-Verbeke
440 Handbook of Chemometrics and Qualimetrics, Elsevier, Netherlands, 1998.
441 [40] M. J. Morilla, P. E. Benavidez, M. O. López, E. L. Romero, J. Chrom. Sci. 41(2003) 405-409.

[41] M. E. Marsón, D. D. Dana, J. Altcheh, F. García-Bournissen, G. Mastrantonio, Therapeutic Drug Monitoring. Send to publish, 2012.

LEGENDS TO THE FIGURES

Fig. 1: Chemical structures of (a) nifurtimox; (b) benznidazole

Fig. 2: Effect of the type of ionic-liquid on extraction efficiency (filled bars, BNZ; empty bars, NFX)

Fig. 3: Effect of the nature of the disperser solvent on extraction efficiency (filled bars, BNZ; empty bars, NFX)

Fig. 4: Effect of the volume of the selected IL on extraction efficiency (■ BNZ, ● NFX).

.

Fig. 5: Effect of the volume of the disperser solvent on extraction efficiency (■ BNZ, ● NFX).

Fig. 6: Effect of pH on extraction efficiency (■ BNZ, ● NFX).

Fig. 7: Effect of the type and concentration of salts on extraction efficiency (■ BNZ, ● NFX).

Fig. 8: Effect of centrifugation and extraction times on extraction efficiency (■ BNZ, ● NFX).

Fig. 9: Chromatograms for a spiked (5 μg mL^{-1}) and a protein-free human-plasma sample before (continued line) and after (dotted line) IL-DLLME preconcentration.

Table 1: Calibration curves for NFX and BNZ

i. Calibration curves in water

Analyte	Linear regression	R*	SD*	N*
BNZ	y= (4.0 ± 2.1) + (2,050,088 ± 11821)x	0.9997	0.2	39
NFX	y= (9.3 ± 4.8) + (4,468,983 ± 28293)x	0.9998	0.5	39

468 **ii. Calibration curves in plasma including DLLME**

Analyte	Linear regression	R*	SD*	N*
BNZ	y= (0.16 ± 0.08) + (2,099,642 ± 10,983)x	0.9993	9.6	24
NFX	y= (1.5 ± 0.1) + (2,722,350 ± 8,790)x	0.9992	22.0	24

469 *R = regression coefficient; SD = standard deviation; N = number of points.

470
471

472 **Table 2:** Limits of detection and quantification obtained for BNZ and NFX in human plasma

473 (concentration units in μg mL^{-1})

474

Compounds	Linear range	LOD[a]	LOD[b]	LOD[c]	LOQ[a]	LOQ[d]
BNZ	0.1323-500.5	0.1009	0.0265	0.0397	0.3058	0.1323
NFX	0.0784-908.7	0.0829	0.0157	0.0235	0.2514	0.0784

475 [a]from calibration curve (IUPAC definition) ; [b]S/N = 2, [c] S/N = 3, [d]S/N = 10.
476

477

478 **Table 3:** Recoveries (%R) and enrichment factors (EF) for human plasma samples at different spiked

479 levels of NFX and BNZ.

480

Spiking level	NFX			BNZ		
(μg mL^{-1})	EF	R(%)[a]	RSD(%)[b]	EF	R(%)[a]	RSD(%)[b]
2.5	38.7	96.8	0.5	31.7	79.4	1.7
5	39.0	97.5	0.2	31.24	78.1	0.1
7.5	39.2	98.1	0.1	31.3	78.2	0.3
10	39.2	98.0	0.1	31.9	79.8	0.6

481 [a] recovery (n = 3), [b] %RSD for recovery.

482
483
484
485

Table 4: Quantitative determinations of NFX and BNZ in spiked human plasma, accuracy and reproducibility (concentrations in μg mL^{-1}).

Analyte	Concentration added	Concentration found[a]	Accuracy (%)	Reproducibility (%RSD)
NFX				
Intra-day	5	4.9 ± 0.2	-2.39	1.79
	7.5	7.3 ± 0.1	-2.98	0.76
	10	9.8 ± 0.2	-2.30	0.97
Inter-day	5	4.9 ± 0.2	-2.27	2.13
	7.5	7.4 ± 0.2	-1.43	1.53
	10	9.8 ± 0.2	-2.30	1.02
BNZ				
Intra-day	5	5.0 ± 0.2	0.025	1.56
	7.5	7.6 ± 0.2	1.61	1.31
	10	10.0 ± 0.5	-0.18	2.52
Inter-day	5	5.0 ± 0.2	0.34	1.59
	7.5	7.6 ± 0.3	1.61	1.89
	10	9.7 ± 0.7	-3.38	3.66

[a] based on 9 levels, each one by triplicate.

497 **Table 5**: Comparison of the method of IL-DLLME-HPLC-UV developed with other procedures described in the
498 literature for the determination of BNZ and NFX in human plasma (NG: not given; WE: no extraction
499 step used).
500

Method	Sample amount (mL)	Extraction solvent (mL)	Volume of extraction solvent (mL)	LR (µg mL^{-1})	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	R%	RSD%[d]	Ref.
BNZ									
HPLC-UV	0.075	WE	WE	1.6–100	0.8	1.6	94.9	1.1	[8]
HPLC-UV	0.2	ACN–DMSO	0.4	0.7–25	NG	0.7[c]	70–97	6.4	[40]
HPLC-UV	NG	WE	WE	0.5–1000	0.2–0.5[b]	NG	≥90	3.2	[13]
HPLC-UV	2.0	ethyl acetate	4.0	0.1–20	0.14[e]	0.3[f]	89	10	[41]
IL-DLLME-HPLC-UV	0.54	[OMIM][PF_6]	0.013	0.1–500	0.04[a]	0.1[c]	78.8	1.3	Our work
NFX									
HPLC-UV	1	CH_2Cl_2	3.5	0.08–2.3	0.08[b]	NG	90.6	3.5	[20]
TLC	10	ethyl acetate	40	0.5–10	0.1-0.2	NG	80	NG	[19]
IL-DLLME-HPLC-UV	0.54	[OMIM][PF_6]	0.013	0.08–908.7	0.02[a]	0.08[c]	97.6	0.76	Our work

501 [a] S/N = 3; [b] S/N = 2; [c] S/N = 10; [d] intra-day; [e] 3.3 s_0; [f] 9 s_0.
502
503

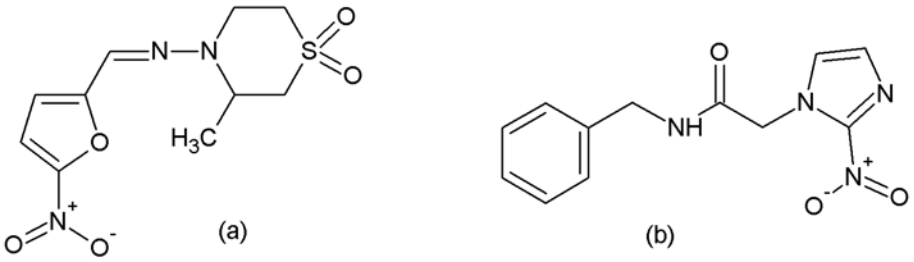

Figure 1

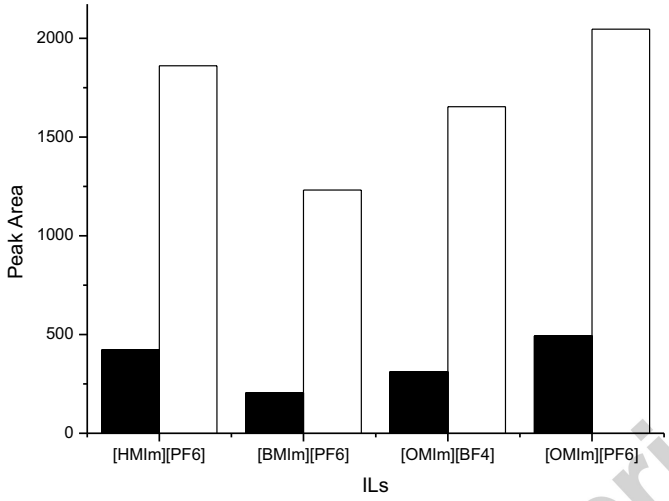

Figure 2

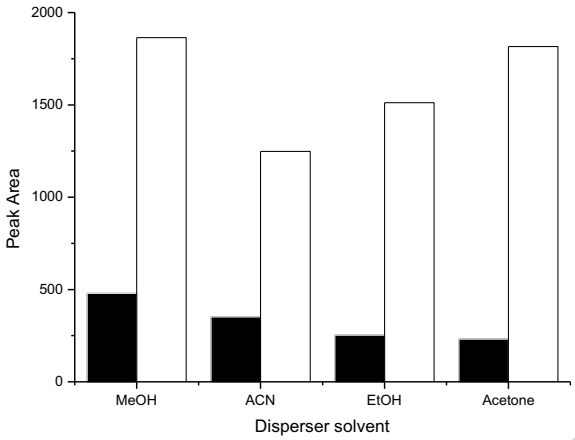

Figure 3

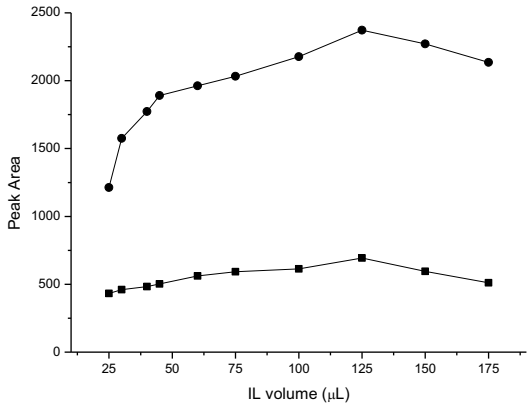

Figure 4

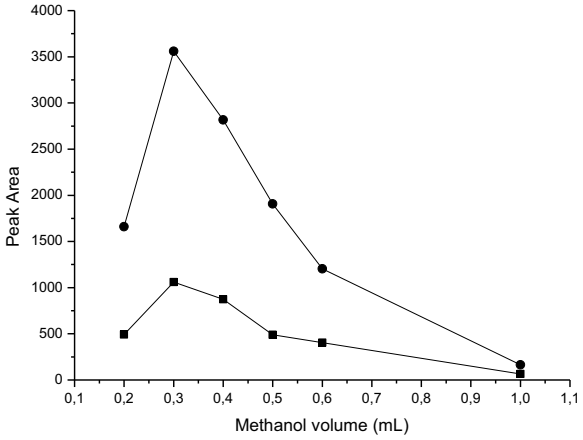

Figure 5

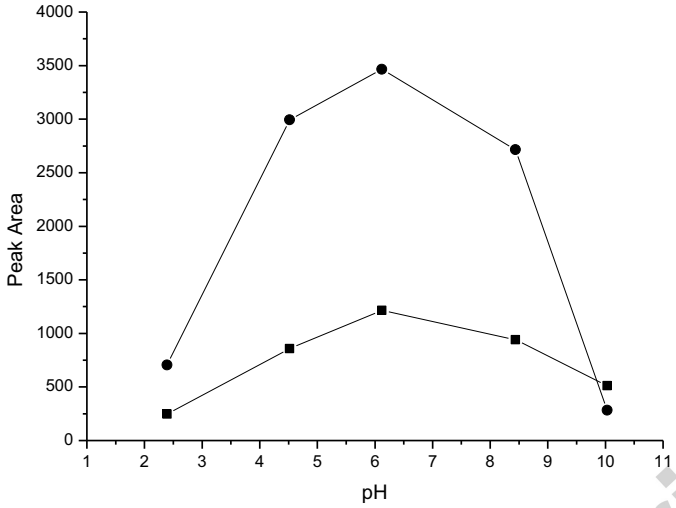

Figure 6

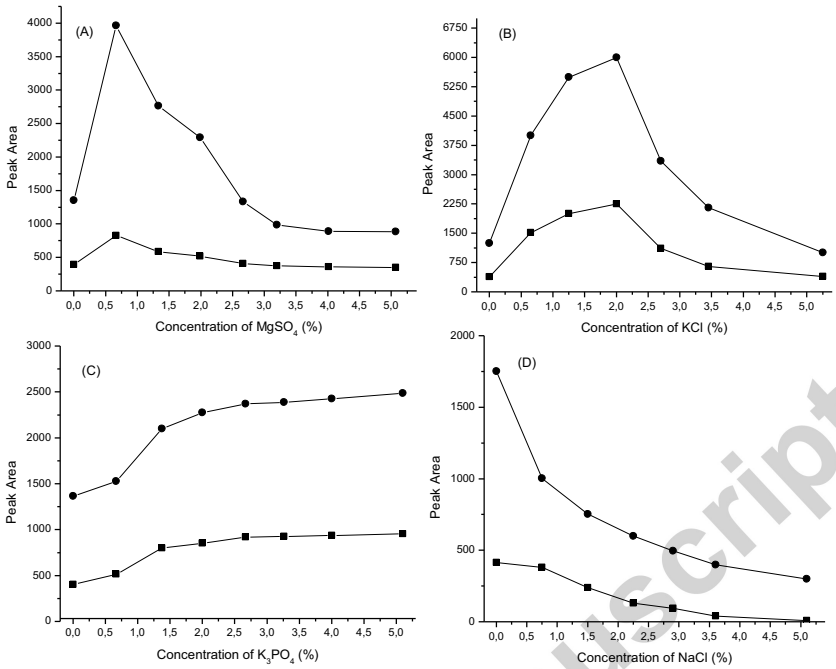

Figure 7

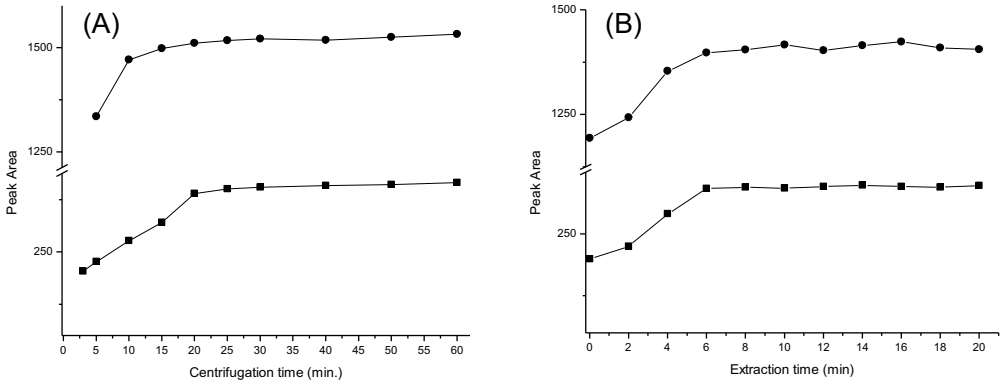

Figure 8

Figure 9

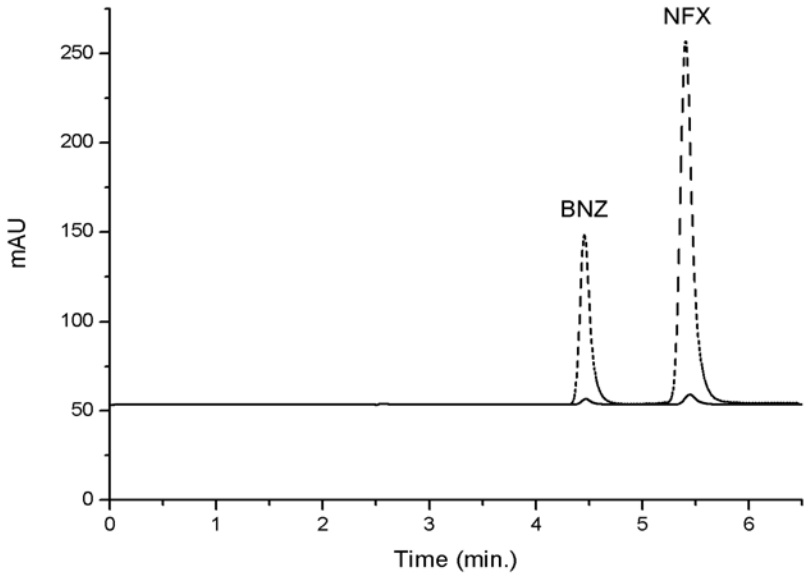

Figure 9