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1

2 **DEVELOPMENT OF AN IONIC LIQUID-BASED DISPERSIVE LIQUID-LIQUID MICROEXTRACTION**

3 **METHOD FOR THE DETERMINATION OF NIFURTIMOX AND BENZNIDAZOLE IN HUMAN PLASMA**

4

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13

14 **Abstract**

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

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

30 Highlights

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

35
36
37

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

40

41 **Introduction**

42 Chagas disease, also known as American trypanosomiasis—first described by Carlos Chagas in 1909
43 [1]—is a potentially life-threatening illness caused by the protozoan parasite *Trypanosoma cruzi (T. cruzi)*.
44 Chagas occurs mainly in Latin America, where transmission to humans is effected either through the feces of
45 triatomine bugs or, in some cases, congenitally [2]. The disease affects approximately 16 to 18 million people,
46 and more than 100 million people are exposed to the risk of infection [3]. In 2008 over 10,000 people were
47 estimated to have died of Chagas disease. Because of the nonvector routes of infection—such as from mother
48 to child, through blood transfusion, or via organ transplantation—the transmission of *T. cruzi* and the disease
49 itself are no longer limited to Latin America, but rather have now become a worldwide problem [4,5]. Chagas
50 disease has been rising in the ranking of international health priorities as a result of the growing extent of
51 migration from endemic to nonendemic areas such as North America and Europe [6,7].

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

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

61 BNZ is a chemotherapeutic drug currently used for the treatment of *T. cruzi* infections in both the
62 chronic and acute phases. A few reports have been published on detection methods for BNZ. Raaflaub and
63 Ziegler [12] investigated the bioavailability of the compound in plasma using polarography. Walton and
64 Workman [13] determined BNZ and its metabolized amine derivative in blood plasma by high-performance
65 liquid chromatography with ultraviolet detection (HPLC-UV) at a recovery of 90% and a reproducibility of
66 3.2%. Barbeira and coworkers have studied direct–current and differential-pulse–polarographic methods for
67 the analysis of BNZ in pharmaceutical formulations [14]. La-Scalea *et al.* investigated the voltametric
68 behavior of BNZ with a glassy-carbon electrode and a DNA-biosensor [15]. The latter enabled the study of
69 BNZ–DNA interactions through the use of immobilized DNA on the glassy–carbon–electrode surface. Only a
70 few authors have developed an HPLC method to quantify BNZ in plasma and/or urine for further
71 implementation in human pharmacokinetic and health-safety studies [12,16-18].

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

78 The determination of clinically significant plasma BNZ or NFX concentrations has generated
79 considerable interest. To our knowledge, two different contexts exist: the plasma concentrations of adults and
80 children. A therapeutic range between 3 and 6 µg mL^{-1} in adult-plasma samples was originally proposed for
81 both drugs on the basis of *in-vitro* data and the results from pharmacokinetic studies in adult humans, but
82 lower values were observed for BNZ in the pediatric patients [21]. Similar values were obtained for NFX
83 [20,22]. Recently, J. Altcheh and colleagues reported a high efficacy of BNZ in pediatric Chagas disease

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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**

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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

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

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

150

151 **2.3. Extraction procedure for the optimization experiments**

152 The IL-DLLME was performed according to the following optimized procedure (see Section 3): 5.00
153 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
154 centrifuge tube. A mixture of 125 µL [OMIM][PF_6] saturated with water and 0.30 mL methanol (the disperser
155 solvent) was injected into the sample solution with a micropipette. After vortex-mixing, a cloudy solution was
156 quickly formed. To increase the extraction efficiency, 0.1 g of KCl had been added. The analytes in the
157 aqueous sample had become extracted into the fine ionic-liquid droplets at this step, while the methanol
158 remained miscible in the aqueous solution. The mixture was then shaken for 6 min and centrifuged at 4,200
159 rpm for 20.0 min. After this centrifuging, the droplets of ionic liquid had completely collected at the bottom
160 of the centrifuge tube. The upper, aqueous phase was removed with a Pasteur pipette without disturbing the
161 underlayer. The IL-phase volume was 120 ± 5 µL. Of the sedimented phase, 5 µL was withdrawn and injected
162 into the HPLC column. All experiments were performed in triplicate.

163

164 **2.4. Extraction procedure for the human-plasma samples**

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

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167 shaken in a vortex for 6 min and centrifuged for 20 min. The supernatant was decanted and filtered through
168 0.22-μm membranes into a 15-mL polypropylene tube.

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

178

179 **2.5. Preparation of stock and standard solutions in water and the sample matrix**

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

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

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

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195 **3. Results and discussion**

196 **3. 1. Optimization of IL-DLLME**

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

205

206 ***3. 1. 1. Selection of the ionic liquid***

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

217 Fig. 2 compares the extraction performance for the four ILs. The extractions were made in triplicate
218 with the same initial volume of the standard solution (5.00 mL) and the same volume of the methanol-IL
219 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]
220 produced the best extraction performance for both antichagasic drugs probably because stronger hydrophobic
221 interactions were established between the longer alkyl chain of the IL and the analytes (Fig. 2). Thus, that IL
222 was used for all of the subsequent experiments.

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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

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251 4.52, sodium phosphate at pH = 6.12, sodium borate at pH = 8.44 and sodium bicarbonate at pH = 10.03), but
252 the ionic strength was kept constant (0.1 M) throughout. A mixture of 0.30 mL of methanol and 125 μL
253 [OMIM][PF_6], was quickly added to the sample solution (Fig. 6). Although, to the best of our knowledge, the
254 relevant pK_a values were not available in the literature, the extraction proved to be maximum at pH = 6.12 so
255 this pH was chosen for the experiments (Fig. 6).
256

257 ***3. 1. 6. Salt effects***

258 The effect of salt addition was determined with four different salts: NaCl, KCl, $MgSO_4$, and K_3PO_4.
259 The extraction efficiency depended on the type and concentration of the salt added (Fig. 7). Previous reports
260 had indicated that when certain salts were used in the aqueous phase for extraction with ILs, the electrostatic
261 interaction between the salt ions and the IL ions enhanced the solubility of the IL in the aqueous phase and
262 thus undermined the extraction efficiency [36-38]. In the present work, this effect was observed for NaCl, but
263 for the other three salts an initial increase in the extraction was obtained as a result of the well known salting-
264 out effect. Furthermore, when KCl was used, the amount of recovered analyte was much higher than with the
265 other salts (*cf*. the *y*-axes in Figs. 7A-D). Since for this salt a maximum was reached at 2.00% (w/v), this
266 concentration of KCl was used.
267

268 ***3. 1. 7. Extraction and centrifugation times***

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

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

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278 plateau (Fig. 8.B). The two solvents obviously required a minimum time to reach equilibrium with the analyte
279 and then separate.
280

281 **3.2. Analytical performance of the proposed methodology**

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

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

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

293 $$t = \frac{b_1 - b_2}{\sqrt{s_{b_1}^2 + s_{b_2}^2}} \qquad (1)$$

294 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
295 standard deviations. If the residual variances s_e^2 for both sets of data are equal (according to an *F*-test), a so-
296 called *combined standard deviation* can be calculated to obtain a *t*-value for comparison with tabulated values
297 for $n_1+n_2–4$ degrees of freedom. If, however, the residual variances are not equal, the Cochran test for the
298 comparison of two slopes with unequal variances must be used. Thus, if s_{b1}^2 is different of s_{b2}^2, then
299 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,
300 respectively, can be obtained from a *t* table. Next, a *combined t′ value* can be calculated by Equation 2, which
301 figure can finally be compared with the calculated *t* values through the use of equation 1:
302

303 $$t' = \frac{t_1 s_{b_1}^2 + t_1 s_{b_2}^2}{s_{b_1}^2 + s_{b_2}^2} \qquad (2)$$

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304 The calculation of t' is not necessary if both regression lines are based on the same number of data points (n_1
305 $= n_2$), in which circumstance $t' = t_1 = t_2$. The working curve in human plasma was compared with the standard
306 one in water to detect matrix effects. For BNZ, the t' value was 2.045, and the t was 3.07; whereas for NFX,
307 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
308 conclude that the slopes were significantly different so that matrix effects were therefore present. As a
309 consequence, for the quantification of BNZ and NFX in plasma samples, the standard addition method was
310 used.

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

315 The lower LOQ in human plasma (at the beginning of the linear range) was evaluated by the S/N of
316 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
317 of quantification) was determined by the lack-of-fit procedure [39]—*i. e.*, by eliminating the highest value
318 and applying the statistical test again with the remaining points. This process is repeated until the data can be
319 adjusted to a straight line.

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

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

328
$$EF = \frac{C_{IL}}{C_{aq}} \qquad (3)$$

329

330 where C_{IL} and C_{aq} are the analyte concentrations in the IL phase and the initial aqueous solution, respectively,
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332 $$R\% = 100\frac{C_{IL}V_{IL}}{C_{aq}V_{aq}} = 100.EF.\varphi \quad (4)$$

333

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

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

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359 The methodology developed and described here is now being applied to the study of real samples—*i.*
360 *e.*, human plasma from infected patients—for future presentation of the findings.
361

362 **3.3. Conclusions**

363 The IL-DLLME–HPLC-UV technique has been used here for the first time to analyze BNZ and NFX
364 in human-plasma samples. The experimental conditions for the extraction of these analytes have been
365 investigated and optimized. Although a step-by-step procedure to obtain the optimum extraction conditions
366 was used, the analytical methodology proved to have several advantages compared to other previously
367 reported extraction techniques—namely, better reproducibility, lower detection limits, and the requirement for
368 much lower amounts of extraction solvent. Moreover, the technique requires very small amounts of sample,
369 which characteristic in the example of human plasma is a highly practical and desirable feature. Recovery was
370 notably high for both compounds, and the performance of the proposed methodology was most satisfactory.
371 Thus, the IL-DLLME-HPLC-UV technique promises to be a simple, fast, efficient, and facile method for the
372 enrichment and quantitative determination of BNZ and NFX in human-plasma samples.
373

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384 **3.5. References**

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444

445 **LEGENDS TO THE FIGURES**

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

447

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

449

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

452 **Fig. 4:** Effect of the volume of the selected IL on extraction efficiency (■ BNZ, ● NFX).
453 .

454 **Fig. 5:** Effect of the volume of the disperser solvent on extraction efficiency (■ BNZ, ● NFX).
455

456 **Fig. 6:** Effect of pH on extraction efficiency (■ BNZ, ● NFX).
457

458 **Fig. 7:** Effect of the type and concentration of salts on extraction efficiency (■ BNZ, ● NFX).
459

460 **Fig. 8:** Effect of centrifugation and extraction times on extraction efficiency (■ BNZ, ● NFX).
461

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

464 **Table 1:** Calibration curves for NFX and BNZ

465

466 **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

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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.

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486
487 **Table 4:** Quantitative determinations of NFX and BNZ in spiked human plasma, accuracy and reproducibility
488 (concentrations in μg mL^{-1}).
489
490

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

491 [a] based on 9 levels, each one by triplicate.
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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 ($\mu g\ mL^{-1}$)	LOD ($\mu g\ mL^{-1}$)	LOQ ($\mu g\ mL^{-1}$)	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.
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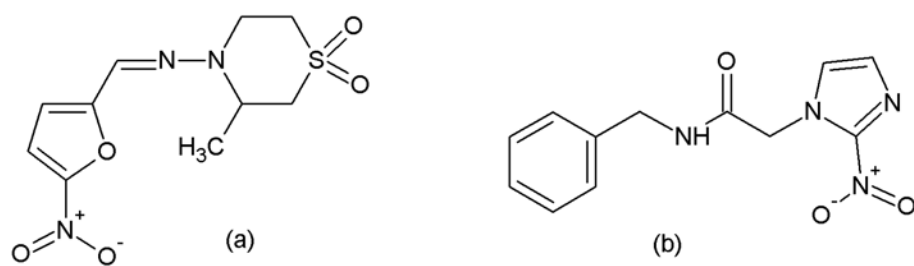

Figure 1

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Figure 2

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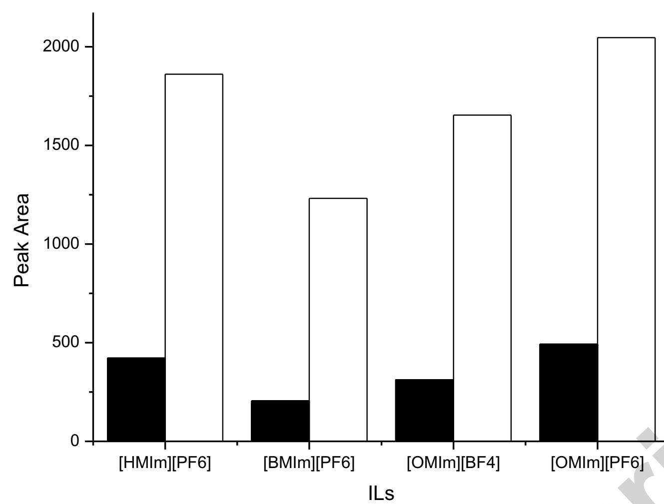

Figure 2

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Figure 3

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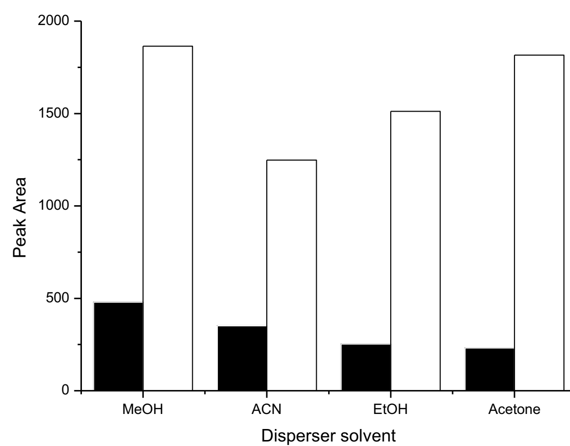

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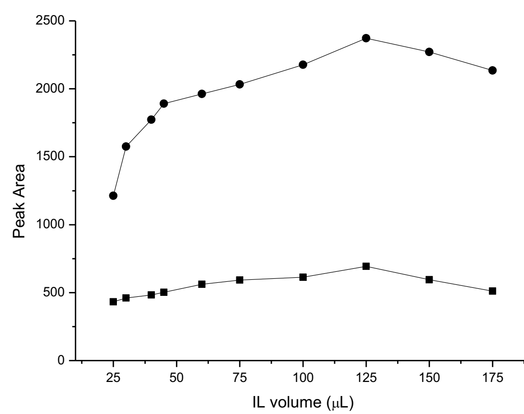

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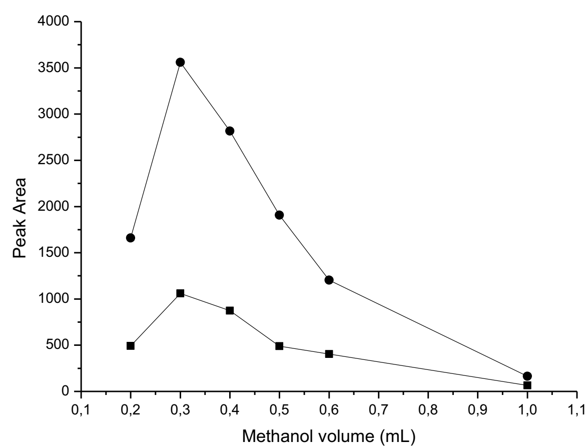

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Figure 6

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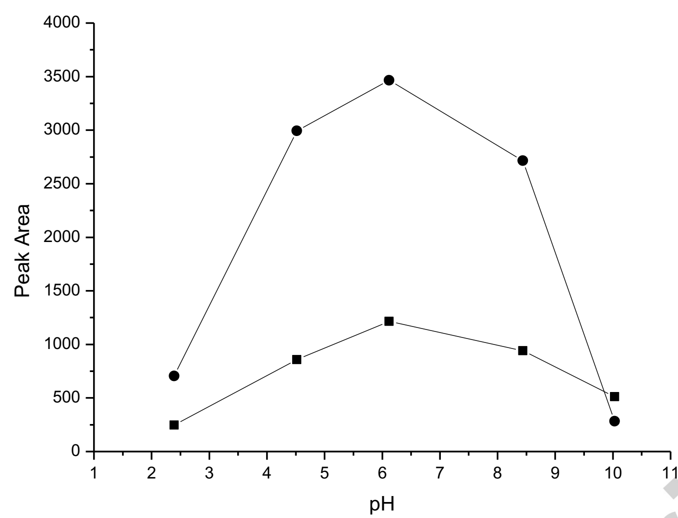

Figure 6

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Figure 7

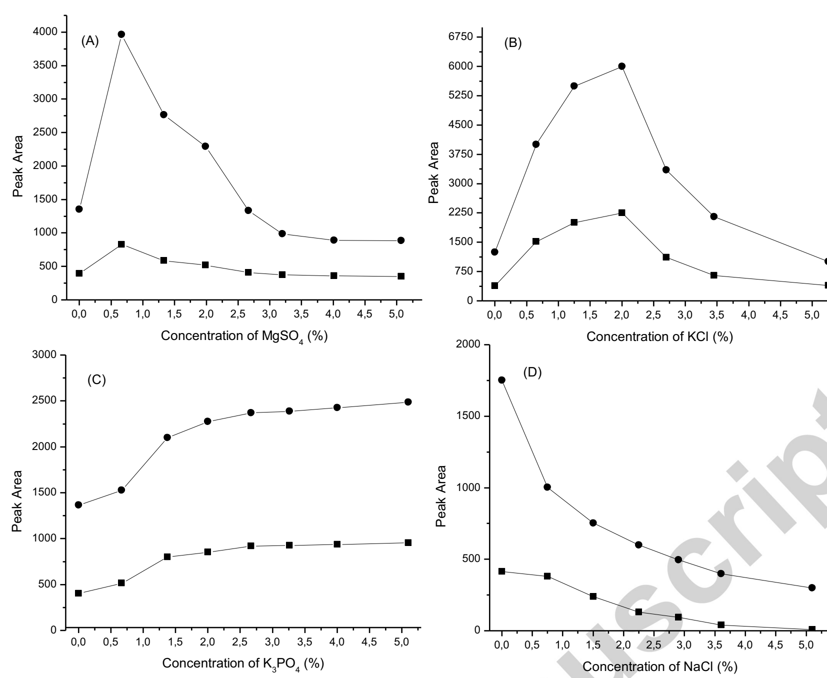

Figure 7

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Figure 8

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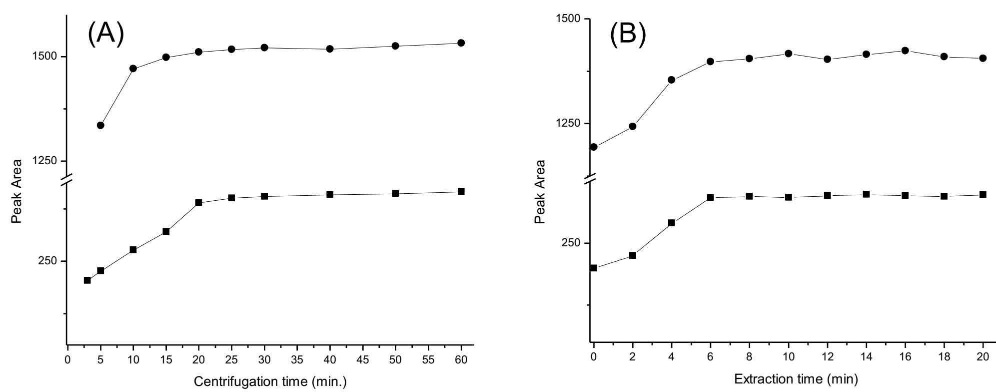

Figure 8

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Figure 9

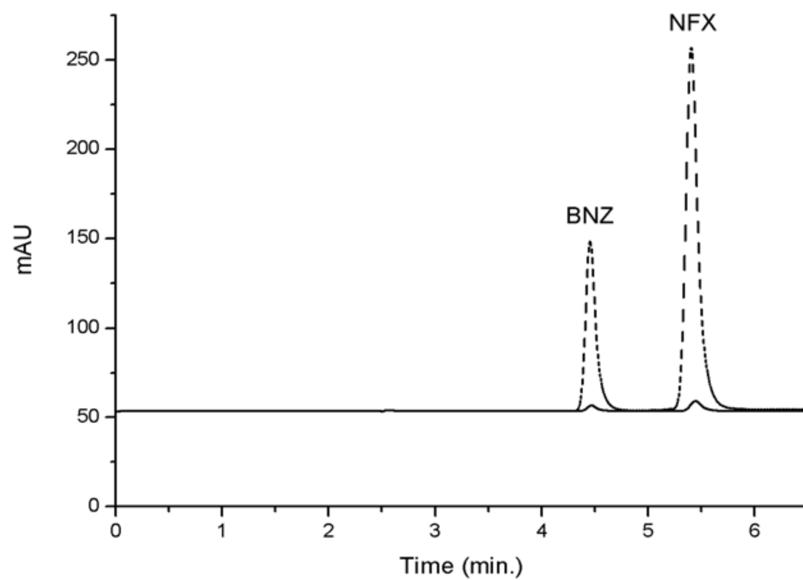

Figure 9