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Yehong Han, Chunliu Yang, Yang Zhou, Dandan Han, and Hongyuan Yan

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1 Ionic Liquid-Hybrid Molecularly Imprinted Material-filter Solid Phase
2 Extraction coupled with HPLC for Determination of 6-Benzyladenine and
3 4-Chlorophenoxyacetic Acid in Bean Sprouts

4 Yehong Han^{1,2}, Chunliu Yang², Yang Zhou², Dandan Han¹, Hongyuan Yan^{1,2*}

5 ¹*Key Laboratory of Medicinal Chemistry and Molecular Diagnosis, College of Public Health,*

6 *Hebei University, Baoding, 071002, China*

7 ²*Key Laboratory of Analytical Science and Technology of Hebei Province & College of Chemistry*

8 *and Environmental Science, Hebei University, Baoding, 071002, China*

9

* Corresponding author. Tel.: +86-312-5079788

E-mail address: yanhongyuan@126.com

10 **ABSTRACT:** A new method involving ionic liquid-hybrid molecularly imprinted
11 material-filter solid phase extraction coupled to high performance liquid
12 chromatography (IL-HIMs-FSPE-HPLC) is developed for simultaneous isolation and
13 determination of 6-benzyladenine (6-BA) and 4-chlorophenoxyacetic acid (4-CPA) in
14 bean sprouts. Sample pre-concentration is performed using a modified filter, with the
15 new IL-HIMs as the adsorbent, which shows double adsorption. The first adsorption
16 involves special recognition of molecular imprinting, and the second one involves
17 ion-exchange and electrostatic attraction caused by the ionic liquid. This method
18 combines the advantages of ionic liquids, hybrid materials, and molecularly imprinted
19 polymers, and is successfully applied to determine 6-BA and 4-CPA in bean sprouts.
20 The adsorption of IL-HIMs to 6-BA is based on selective imprinted recognition, while
21 the adsorption of 4-CPA is mainly dependent on ion-exchange interactions.

22 **KEYWORDS:** *ionic liquid hybrid molecularly imprinted polymer, 6-benzyladenine,*
23 *4-chlorophenoxyacetic acid, filter solid phase extraction, bean sprouts*

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26 **INTRODUCTION**

27 6-Benzyladenine (6-BA) is a common cytokinin, one of the important classes of plant
28 hormones, which is widely used to promote seed germination and nursery trees.¹
29 4-Chlorophenoxyacetic acid (4-CPA) is a widely used chlorophenoxy herbicide and
30 plant growth regulator in agriculture and is considered as moderately toxic (class II or
31 III) by the World Health Organization.^{2,3} 6-BA and 4-CPA are widely applied to
32 promote stalk growth and restrict root growth in the production process of bean
33 sprouts. Thus, bean sprouts contain 6-BA and 4-CPA residues that could be hazardous
34 to human health in the long term. Therefore, the maximum residue limits (MRLs) for
35 4-CPA (1.0 mg/kg) and 6-BA (0.2 mg/kg) in bean sprouts have been established by
36 Chinese government.⁴ In recent years, numerous poisoning incidences caused by bean
37 sprouts containing 6-BA and 4-CPA have been reported in China. The quantitative
38 analysis of 6-BA and 4-CPA in bean sprouts is difficult because these phytohormones
39 are present in low concentrations and the tissues are complicated as they contain lots
40 of interfering substances. In order to monitor the trace levels of 6-BA and 4-CPA, a
41 sensitive, accurate, and robust analytical method is desired.

42 Up until now, the approaches for the determination of 6-BA and 4-CPA include gas
43 chromatography (GC),⁵ high performance liquid chromatography (HPLC),^{6,7} and
44 voltammetric methods.⁸ In order to isolate and concentrate the analytes prior to
45 instrumental analysis, a clean-up and pre-concentration step is desired. Some routine
46 sample pre-treatment techniques such as liquid-liquid extraction (LLE),⁹ solid-phase
47 extraction (SPE),¹⁰ and dispersive liquid-liquid microextraction¹¹ have been proposed

48 for extraction and isolation of 6-BA and 4-CPA. Recent trends in sample pre-treatment
49 technology are oriented toward high selectivity, miniaturization, and low consumption
50 of reagents and equipment. The extractant/adsorbent is an important factor in most
51 pre-treatment techniques, especially for SPE, which significantly affect the extraction
52 efficiency. However, common adsorbents (such as C₁₈, C₈, silica, hydrophile-lipophile
53 balance, strong cation exchanger, etc) was lack of good selectivity and common
54 pre-treatment techniques were faced with the difficulty of extracting trace amounts of
55 the analytes from complicated sample matrices; therefore, development of selective
56 adsorbent materials for sample pre-treatment procedures is required.

57 Molecular imprinting is an attractive strategy for generating tailor-made polymers,
58 containing molecular recognition sites with memory of the shape, size, and functional
59 groups of template molecules.¹² Molecularly imprinted polymers (MIPs) have been
60 increasingly used for the isolation and efficient separation of trace compounds from
61 complex matrices due to their high selectivity, reusability, and physicochemical
62 stability in harsh chemical media.^{13,14} However, these materials may shrink or swell
63 when exposed to different organic solvents and change the morphology of the
64 polymer network, causing considerable deformation of the MIP receptors and
65 decreasing the recognition ability toward the template.^{15,16} Common MIPs are
66 normally compatible with organic solvents and fail to show specific binding in
67 aqueous solution,¹⁷ which significantly limits their practical application as adsorbents
68 and biomimetic sensors.¹⁸ Recently, organic-inorganic hybrid MIPs are developed,
69 which offer high mechanical strength, good solvent resistance, and high stability in

70 different pH environments. They have been widely applied for environmental
71 monitoring and food analysis, because of their attractive properties.¹⁹ However, most
72 hybrid MIP bonding analytes depend on hydrogen bonding and other hydrophobic
73 interactions, which limit their further application in the fields of environmental
74 science and biology. Herein, extensive studies have been carried out on hybrid MIPs
75 with specific molecular recognition ability in aqueous solution.

76 Recently, ionic liquids (ILs) as alternative green solvents, have received a great
77 deal of attention as the surface modifier of polymers or functional monomers, because
78 they can provide ion-exchange interactions, electrostatic interactions, π - π interactions,
79 etc. ILs maintain their properties, except outflow, when they immobilize on a silica
80 surface or an organic polymer; therefore, IL-modified materials have been used in
81 separation owing to their particular characteristics such as their electronegative group
82 relay on ion exchange, electrostatic interaction, and π - π interaction between the
83 analyte and functional groups of ILs.²⁰⁻²⁴

84 In this study, a new type of ionic liquid-hybrid molecularly imprinted material
85 (IL-HIMs) was synthesized using 1-allyl-3-propyltrimethoxysilane imidazole as both
86 the monomer and surface modifying agent. The synthesized IL-HIM is a double
87 adsorption mechanism material, which not only can interact with a template and its
88 structural analogs based on the special recognition of molecular imprinting, but also
89 absorbs analytes with electronegative groups by ion-exchange, electrostatic, and π - π
90 interactions. Owing to their synergic effect, the novel IL-HIMs show good selectivity,
91 and they can be used for various purposes as they show a variety of adsorption

92 mechanisms, have no swelling and shrinking, and have a specific affinity for analytes
93 in aqueous solutions. IL-HIMs are successfully applied as the adsorbent using filter
94 solid-phase extraction, resulting in rapid extraction and isolation of 6-BA and 4-CPA
95 in bean sprouts.

96 MATERIALS AND METHODS

97 **Materials.** 6-Benzyladenine (6-BA), 2, 4-chlorophenoxyacetic acid sodium (2,4-D),
98 4-chlorophenoxyacetic acid sodium (4-CPA), acyclovir (ACV), kinetin (KT), and
99 indoleacetic acid (IAA) were purchased from Jingchun Co., Ltd. (Shanghai, China)
100 and their molecular structure were shown in Figure 1. 1-Vinylimidazole, ethylene
101 glycol dimethacrylate (EGDMA), 3-chloropropyltrimethoxy silane (CPTMO),
102 tetraethyl orthosilicate (TEOS), and methacrylic acid (MAA) were purchased from
103 Aladdin reagent Co., Ltd. (Shanghai, China). MAA and EGDMA were used after
104 vacuum distillation. Azodiisobutyronitrile (AIBN) was purchased from Yuefeng
105 Chemical Co., Ltd. (Tianjin, China) and recrystallized before use. Methanol (MeOH),
106 ethanol (EtOH), acetone, acetonitrile (ACN), and acetic acid (AA) were purchased
107 from Guangfu chemical Co., Ltd. (Tianjin, China). Bean sprouts were bought in the
108 local markets of Baoding, China. The commercial C₁₈ and amino (NH₂) sorbent were
109 supplied by Varian Co. (Palo Alto, CA, USA). All the other reagents used in the
110 experiment were of the highest grade commercially available.

111 **Instrumentations and Conditions.** A FTIR-8400S Fourier transform infrared
112 (FTIR) spectrometer (Shimadzu, Kyoto, Japan) was used to examine the infrared
113 spectra of the obtained materials in a range of 400-4000 cm⁻¹. The morphological

114 evaluation was performed by JSM-7500F Scanning electron microscopy (JEOL Ltd,
115 Tokyo, Japan). Nitrogen adsorption-desorption measurements were performed on a
116 Tristar II 3020 Pore Size and Surface Area Analyzer (Micromeritics, Norcross, USA).
117 The surface-area was measured on Brunauer-Emmett-Teller (BET), and the pore-size
118 distribution was based on Barrett-Joyner-Halenda (BJH) formula. Chromatographic
119 analysis was carried out on a LC-20A system equipped with two LC-20AT Solvent
120 Delivery Units, a SUS-20A gradient controller, and a SPD-20A UV-VIS Detector
121 (Shimadzu, Kyoto, Japan). The Ultimate AQ-C₁₈ column (4.6×150 mm, 5 μm) was
122 purchased from Welch Materials, Inc., and the mobile phase was methanol-water (1:1,
123 v/v, containing 1% acetic acid) with a flow rate of 1.0 mL/min. The wavelength of the
124 detector was set at 267 nm (0-8 min) and 228 nm (8-12 min).

125 **Synthesis of the IL-HIMs.** Allylimidazole (3.5 mmol) was added dropwise to a
126 conical flask containing 3-chloropropyltrimethoxysilane (CPTMO, 2.5 mmol),
127 sonicated for 10 min and purged with N₂ for 20 min. Then, the flask was sealed and
128 heated at 80 °C using an oil bath for 24 h. The viscous liquid obtained was washed
129 with ether and dried under vacuum to obtain the desired IL (1-allyl-3-propyl-
130 trimethoxysilane imidazolium chloride).

131 KT (0.5 mmol) and MAA (2.0 mmol) were dissolved in 10.0 mL of acetonitrile
132 (ACN) in a conical flask containing 1-allyl-3-propyl-trimethoxysilane imidazolium
133 chloride. The mixture was sonicated for 10 min and stored at 4 °C in darkness.
134 EGDMA (25.0 mmol) and AIBN (40 mg) were added to the conical flask and
135 sonicated for 10.0 min. This was then added to a flask containing TEOS (4.48 mmol)

136 after alcoholysis. The mixture was degassed by sonication and subjected to thermal
137 initiation at 60 °C for 24 h in a water bath. After polymerization, the IL-HIMs were
138 washed with a methanol-acetic acid mixture (90:10, v/v) and methanol successively to
139 remove the template, and they were then dried under reduced pressure (Figure 2). The
140 non-ionic liquid-hybrid imprinted material (NIL-HIMs) and non-ionic
141 liquid-hybrid-non-imprinted material (NIL-HNIMs, without IL and template) were
142 prepared using an identical procedure.

143 **Adsorption Capacity of IL-HIMs.** Firstly, static adsorption experiments were
144 carried out, in the following steps. (1) Each IL-HIM, NIL-HIM, and NIL-HNIM
145 adsorbent (50 mg) was added into an empty cartridge and pre-treated first with 1.0 mL
146 of methanol and then with 1.0 mL of water. The solution containing 6-BA and 4-CPA
147 was loaded onto the cartridge discontinuously, at 1 mL intervals. The concentration of
148 the mixed solution was 10 (1-3 mL) and 30 µg/mL (4-n mL). The concentration of
149 6-BA and 4-CPA in the effluent was determined by HPLC. (2) Each IL-HIM,
150 NIL-HIM, and NIL-HNIM filter was placed in 2.0 mL aqueous solutions containing
151 40 µg/mL of 6-BA and 4-CPA. The solutions were shaken in a water bath at 25 °C and
152 analyzed at 0.5, 1, 3, 5, 8, 9 and 15 h to measure the concentration of 6-BA and 4-CPA
153 using HPLC.

154 A competitive adsorption experiment was also performed by placing each IL-HIM,
155 NIL-HIM, and NIL-HNIM adsorbent into a 2.0 mL solution containing 0.2 mmol/mL
156 of 6-BA, KT, 4-CPA, ACV, 2,4-D, and IAA. The concentrations of the different
157 compounds after adsorption were obtained using HPLC by means of standard curves.

158 **Analysis of Bean Sprouts.** Bean sprouts were purchased from local supermarkets
159 in Baoding, China. Sprout samples (500 g) were compressed using a juice extractor,
160 and the juice was collected and centrifuged at 4000 rpm for 15 min. The residue was
161 re-extracted using a methanol-acetic acid mixture (9:1, v/v). After removing the
162 solvent by reduced pressure distillation, the extract was re-dissolved using the sprout
163 juice for filter solid-phase extraction (FSPE).

164 The IL-HIM adsorbent (40 mg) was placed into a replaceable film needle filter. The
165 process of the FSPE (Figure 3A) coupled to HPLC, for extraction and determination
166 of 6-BA and 4-CPA, was as follows: Firstly, the filter was conditioned using methanol
167 (1.0 mL) and water (1.0 mL) at a flow rate of 0.2 mL/min and then the sprout juice
168 sample was loaded onto the filter using a syringe at a flow rate of 0.2 mL/min. After
169 washing with 0.8 mL of water, the filter was subsequently flushed with 3.0 mL of
170 methanol-AA (9:1, v/v), at a flow rate of 0.2 mL/min. The eluted fractions were
171 collected, evaporated to dryness, and then reconstituted in 0.5 mL of the mobile phase
172 for HPLC analysis.

173 **RESULTS AND DISCUSSION**

174 **Preparation of IL-HIMs.** The aim of this work was to produce an adsorbent
175 material for the extraction and isolation of 6-BA and 4-CPA. The new IL was
176 synthesized following a reported method^{25,26} with some modifications, making use of
177 both surface-modifying agents and monomers in the polymerization process,
178 promoting stronger conglutination of the outer organic polymer onto the silica matrix.
179 The C=C of the IL can be cross-linked with EGDMA or MAA, and the silicate ester

180 bond can be embedded in the silica matrix after condensation. MAA is also used as a
181 monomer in the polymerization, and the proportion of MAA and IL was investigated.
182 The adsorbent synthesized using IL without MAA showed 51% recovery of 6-BA, the
183 adsorbent synthesized using MAA only gave a recovery of below 10% for 4-CPA, and
184 MAA:IL (2:2, n/n) exhibited over 95% recovery for both 4-CPA and 6-BA. The
185 adsorption of 4-CPA is mainly dependent on π - π and ion-exchange interactions
186 between the IL and 4-CPA, while the adsorption of 6-BA is mainly dependent on
187 hydrogen bonding between -COOH and =N- or -NH-; therefore, the IL and MAA
188 were both chosen as monomers. EGDMA is the cross linker, and the -C=C- of
189 EGDMA can interact with the -C=C- of MAA and IL by atom-transfer
190 radical-polymerization. TEOS can interact with the $-\text{Si}(\text{OH})_3$ of the IL by
191 polycondensation. For the synthesis of the outer organic polymer under thermal
192 initiation, acetonitrile is a good porogenic agent, and its use resulted in an adsorbent
193 with a satisfactory binding capacity. After optimization, 30 mL acetonitrile was added
194 to the reaction mixture.

195 **Characterization of the Materials.** The scanning electron microscopy (SEM)
196 images in Figure 3B revealed that the IL-HIM (a) has a rough surface and a loose,
197 porous structure, which leads to a higher surface area and is beneficial for the
198 adsorption rate. Figure 3C shows the infrared (IR) spectrum of the IL-HIMs. The band
199 at 1160 cm^{-1} is assigned to the asymmetric stretching vibration, Si-O-Si, and at 1650
200 cm^{-1} a band corresponding to the C=C and C=N vibrations is observed.²⁷ A band
201 observed at 1725 cm^{-1} is attributed to the C=O vibration of the EGDMA. Absorption

202 bands at 2995 cm^{-1} and 960 cm^{-1} show the presence of the $-\text{COOH}$ moiety in this
203 polymer. At the same time, the absorption band at 3400 cm^{-1} shows that the materials
204 possess abundant $-\text{SiOH}$ moieties to afford the adsorption capacity.²⁸ The band at
205 1459 cm^{-1} corresponding to the vibration of imidazole ring indicated that the
206 vinylimidazolium IL had been incorporated into the polymers.

207 The results of BET showed that the specific surface area of the IL-HIMs (403.5
208 m^2/g) were larger than those of the NIL-HIM ($187.4\text{ m}^2/\text{g}$) and NIL-HNIMs (172.8
209 m^2/g), which resulted from more porous and rough surface of IL-HIMs caused by IL
210 (acting as porogenic solvent and stabilizers for designing nanoporous structures) and
211 template molecules (more pores remained after the removal of the template). The data
212 of the desorption pore size distribution of the IL-HIMs and NIL-HNIMs show that the
213 pore diameter of the IL-HIMs is mainly distributed between 5 and 50 nm and the
214 NIL-HNIMs between 5 and 30 nm.

215 To evaluate the binding property of the IL-HIMs, NIL-HIMs, and NIL-HNIMs,
216 static adsorption and dynamic adsorption experiments were performed. The results
217 (Figure 4A) of the static adsorption experiment show that the adsorption of 4-CPA
218 increased with an increase in concentration, and the adsorption capacities gradually
219 tended to be saturated when the concentration of 4-CPA was more than $275\text{ }\mu\text{g/mL}$.
220 Compared with NIL-HIMs and NIL-HNIMs, IL-HIMs revealed a higher adsorption
221 capacity owing to the electrostatic and ion-exchange interactions of the IL and π - π
222 interactions between the imidazole ring of the IL and the benzene ring of 4-CPA. Due
223 to the large difference in the molecular structure between the imprinted template and

224 4-CPA, there are no specific imprinted recognition interactions between the materials
225 (IL-HIMs and NIL-HIMs) and 4-CPA. Figure 4B shows that the adsorption capacity
226 of the IL-HIMs and NIL-HIMs to 6-BA was significantly higher than that of the
227 NIL-HNIMs, owing to specific imprinted recognition. The adsorption capacities of
228 the IL-HIMs for 6-BA were higher than those of the NIL-HIMs because of the
229 electrostatic and π - π interactions between the IL and 6-BA.

230 To investigate the equilibrium time, the effect of contact time was studied at 25 °C
231 in aqueous media with an initial 6-BA and 4-CPA concentration of 0.25 mmol/L. The
232 dynamic adsorption (Figure 4C) of 6-BA shows that both the IL-HIMs and NIL-HIMs
233 were balanced at nearly 60 min, which indicates that the interaction of 6-BA and
234 IL-HIMs was similar to that of 6-BA and NIL-HIMs, mainly owing to imprinted
235 recognition. The adsorption equilibrium showed that the mass transfer rate of the
236 IL-HIMs and NIL-HIMs became better than that of the NIL-HNIMs. From the results
237 presented in Figure 4D, it can be deduced that the adsorption capacity of the IL-HIMs
238 for 4-CPA increased with an increase in time and reached the equilibrium value at 90
239 min. The equilibrium value of the NIL-HIMs and NIL-HNIMs had a lower adsorption
240 capacity than the IL-HIMs for 4-CPA under the same conditions. This partially
241 accounted for the higher surface area of the IL-HIMs compared with that of the
242 NIL-HIMs and NIL-HNIMs (as confirmed by BET analysis). Obviously, through
243 electrostatic, ion-exchange, and π - π interactions, the adsorption of 4-CPA was greatly
244 enhanced.

245 **Selectivity and Competitive Experiments.** A mixed solution containing 6-BA,

246 4-CPA, KT, 2,4-D, ACV, and IAA was analyzed to evaluate the selectivity and
247 competitive adsorption of the IL-HIMs, NIL-HIMs, and NIL-HNIMs. Figure 5 shows
248 that the adsorption capacities of 6-BA for the IL-HIMs and NIL-HIMs were
249 significantly higher than that of the NIL-HNIMs. The IL-HIMs, NIL-HIMs, and
250 NIL-HNIMs had a low adsorption capacity toward ACV. It was further certified that
251 6-BA was adsorbed onto the IL-HIMs by means of specified imprinted sites, but other
252 compounds were adsorbed mainly by non-specified sites on the IL-HIMs. However,
253 the IL-HIMs could adsorb much more 2,4-D, 4-CPA, and IAA than the NIL-HIMs and
254 NIL-HNIMs, mainly because the $-\text{COOH}$ (2,4-D, 4-CPA, and IAA) and $-\text{Cl}$ (2,4-D
255 and 4-CPA) moieties can effectively interact with the IL-HIMs by electrostatic and
256 ion-exchange interactions.

257 **Optimization of IL-HIMs-FSPE Procedures.** The developed filter solid-phase
258 extraction (FSPE), a miniaturized SPE method, uses a modified needle filter instead of
259 common SPE cartridges and has many advantages, mainly including small device,
260 rapid and facile operation, and low consumption of sorbent and solvent. To achieve
261 the highest recoveries of trace levels of 6-BA and 4-CPA from bean sprout samples,
262 several parameters including the type of washing solvent, volume of washing solvent,
263 and elution solvent for the IL-HIMs-FSPE system were investigated.

264 The washing step played a crucial role in removing interferences originating from
265 sample matrices, while maintaining specific interactions between the analytes and
266 IL-HIMs. Different washing solvents such as MeOH-H₂O (1:9, v/v), ACN-H₂O (1:9,
267 v/v), acetone-H₂O (1:9, v/v), MeOH, ACN, water and acetone were investigated (1.0

268 mL spiked sample (3.0 $\mu\text{g}/\text{mL}$), 1.0 mL of each solvent), and the results were shown
269 in Figure 6A. Using MeOH-H₂O (1:9, v/v), ACN-H₂O (1:9, v/v), and water as the
270 washing solvent, provided improved recoveries compared to MeOH, acetone,
271 acetone-H₂O (1:9, v/v), and ACN. Meanwhile, the recoveries obtained when water
272 was used were slightly higher than those obtained when MeOH-H₂O (1:9, v/v) and
273 ACN-H₂O (1:9, v/v) were used as the solvents. Considering that water is an
274 environmentally friendly solvent and its purification efficiency is similar to that of
275 MeOH-H₂O (1:9, v/v) and ACN-H₂O (1:9, v/v), water was chosen as the washing
276 solvent. Various volumes (0.5, 0.8, 1.0, 1.2, and 1.5 mL) of water were investigated to
277 improve the purification with minimal loss of 6-BA and 4-CPA. The results (Figure
278 6A) indicate that the recoveries and purification efficiency were satisfactory if the
279 volume of water was increased to 0.8 mL, whereas the recoveries gradually decreased
280 if the volume was more than 0.8 mL. Hence, 0.8 mL of water was selected as the
281 optimal volume.

282 Various types of elution solvents were investigated, including water-AA (90:10,
283 v/v), methanol-AA (95:5, 90:10, 85:15, v/v), acetone-AA (10:90, v/v), ethanol-AA
284 (10:90, v/v), and acetonitrile-AA (10:90, v/v), to ensure complete analyte desorption
285 from the IL-HIMs. Figure 6B shows the elution efficacy in terms of recoveries for
286 6-BA and 4-CPA. The highest elution efficiency was achieved using methanol-AA
287 (95:5, v/v) as the elution solvent, which suggested that 6-BA and 4-CPA were bound
288 to the binding sites of the IL-HIMs, possibly through hydrogen bonds and other
289 interactions, which are affected and altered by polar solvents and acid, allowing for

290 rapid desorption. After optimization of the elution volume, 3.0 mL methanol-AA (95:5,
291 v/v) was selected as the elution volume for the IL-HIMs-FSPE.

292 **Comparison of Different Adsorbents.** To further demonstrate the characteristics
293 of the IL-HIMs, various adsorbents including IL-HIMs, NIL-HNIMs, C₁₈, and NH₂
294 were investigated for the FSPE. The loading, washing, and elution conditions of C₁₈
295 and NH₂ were modified from literature.⁶ The conditions of the IL-HIMs and
296 NIL-HNIMs for FSPE were constant, based on two adsorbents prepared in an
297 identical manner. As shown in Figure 7, the recoveries of 6-BA were less than 78.2%
298 when using C₁₈, NH₂, and NIL-HNIMs as the adsorbents. Moreover, the recoveries of
299 4-CPA using C₁₈ and NIL-HNIMs were 56.6% and 33.4%, respectively. Conversely,
300 the IL-HIMs yielded the highest recovery of 6-BA (94.5%) and 4-CPA (96.4%) with
301 obviously improved purification efficiency, which indicates a high selectivity and
302 affinity of the IL-HIMs for 6-BA than that of the other adsorbents. Meanwhile, the
303 recovery (90.2%) from the NH₂ adsorbent for 4-CPA was a little lower than that of the
304 IL-HIMs, which indicates that the -NH₂ group can interact with 4-CPA by
305 ion-exchange similar to the IL-HIMs.

306 **Validation of the IL-HIMs-FSPE-HPLC-UV Method.** The developed method
307 was evaluated for linearity, precision, accuracy, limits of detection (LOD) and limits
308 of quantification (LOQ) under the optimized conditions. The calibration curve was
309 constructed using spiked bean sprout samples with 6-BA and 4-CPA in the range of
310 0.2-4.8 µg/mL. The calibration equations (peak height vs. analyte concentration) for
311 6-BA and 4-CPA are $Y = 2.8 \times 10^{-5}X - 128.46$ and $Y = 1.7 \times 10^{-5}X + 2464.7$ with

312 correlation coefficients of $r \geq 0.9992$ and 0.9994 , respectively. The precision of the
313 method was evaluated with relative standard deviation (RSD) of reproducibility
314 (intraday and interday precision), and the reproducibility was determined every three
315 months. Adequate precision was found and intraday RSD values for 6-BA and 4-CPA
316 were less than 2.6% and 1.6%, respectively. The interday RSD values for 6-BA and
317 4-CPA were less than 7.6% and 3.9%, respectively. The LOD and LOQ values ($n = 3$)
318 (calculated at signal-to-noise ratios of 3 and 10, respectively) of 6-BA were 0.04 and
319 $0.13 \mu\text{g/mL}$ and those of 4-CPA were 0.055 and $0.18 \mu\text{g/mL}$, respectively. To
320 demonstrate the effect of the sample matrix, the accuracy of the proposed method was
321 assessed by recovery experiments at three spiking levels (0.2 , 2.0 , and $4.0 \mu\text{g/mL}$) of
322 6-BA and 4-CPA. The recoveries of 6-BA and 4-CPA were in the range
323 88.6% - 106.2% with $\text{RSD} \leq 7.6\%$ ($n=3$) and 81.1% - 106.1% with $\text{RSD} \leq 3.9\%$,
324 respectively, which indicated that the IL-HIMs-FSPE-HPLC-UV method was reliable
325 and could be used for simultaneous determination of 6-BA and 4-CPA in bean sprouts
326 (Table 1).

327 The comparison of the presented method with reported methods for the extraction
328 of 6-BA and 4-CPA has been shown in Table 2. Most reported methods involving the
329 determination of only one of the two target substances. Compared with these reported
330 methods, the developed IL-HIMs-FSPE-HPLC method can detect 6-BA and 4-CPA
331 simultaneously with the advantages of high recovery and low LOD.

332 **Analysis of Bean Sprouts.** Twenty kinds of bean sprout samples collected from the
333 local markets of Baoding were analyzed to evaluate the applicability of the proposed

334 method. The results showed that one of the bean sprout samples contained 6-BA at
335 levels of 0.18 $\mu\text{g/g}$ (MRL < 0.2 $\mu\text{g/g}$). Another sample was observed to contain 4-CPA
336 at levels of 0.36 $\mu\text{g/g}$ (MRL < 1.0 $\mu\text{g/g}$). The chromatograms of the spiked bean
337 sprout were cleaner after the IL-HIMs-FSPE process, as seen in Figure 8, and there
338 were no interfering peaks originating from the bean sprout matrix. Hence, it can be
339 concluded that the IL-HIMs-FSPE-HPLC-UV method is reliable and can be used for
340 simultaneous determination of 6-BA and 4-CPA in bean sprouts.

341

342 AUTHOR INFORMATION

343 Corresponding Author

344 * Tel.: +86-312-5079788. E-mail: yanhy@hbu.edu.cn.

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350 Notes

351 The authors declare no competing financial interest.

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- 452

453 **FIGURE CAPTIONS**

454 **Figure 1.** Molecular structure of 6-BA, 4-CPA and some analogues.

455 **Figure 2.** Schematic illustration of IL-HIMs preparation.

456 **Figure 3.** The process of FSPE (A) and the SEM (B) and FTIR (C) of IL-HIMs.

457 **Figure 4.** Loading isotherm of analytes on IL-HIMs, NIL-HIMs, and NIL-HNIMs.

458 (A and B: static adsorption; C and D: dynamic adsorption)

459 **Figure 5.** The selectivity of IL-HIMs, NIL-HIMs, and NIL-HNIMs.

460 **Figure 6.** Effect of washing and eluent solvents on the recovery of IL-HIMs-FSPE.

461 (A: washing solvents; B: eluent solvents)

462 **Figure 7.** The purification effect (A) and recovery (B) of different adsorbents.

463 **Figure 8.** Chromatogram of spiked sample (A, B) and real samples (C, D).

TABLES

Table 1. Recoveries of 6-BA and 4-CPA in spiked samples (n=3).

Spiked level	0.2 µg/mL		2.0 µg/mL		4.0 µg/mL	
Analytes	Recoveries (%)	RSD (%)	Recoveries (%)	RSD (%)	Recoveries (%)	RSD (%)
6-BA	92.1	3.5	106.2	1.5	95.4	1.4
6-BA	96.3	4.0	100.5	2.3	88.6	5.4
6-BA	94.2	7.6	105.3	1.1	103.8	5.8
4-CPA	81.1	2.1	106.1	1.7	100.2	1.1
4-CPA	86.9	2.4	103.5	1.3	101.9	3.9
4-CPA	83.9	1.2	104.8	1.2	98.4	2.0

Table 2 Comparison of the presented method with reported methods and standard methods.

Samples	Pretreatment	Detection	Linearity	LOD	Recovery (%)	RSD (%)	Ref.
Apple	SPE	HPLC-UV	0.1–200 µg/g	10.6 ng/g	77.2–89.4	≤2.22	29
Bean sprouts	-	icELISA	3.6–106 ng/mL	18.9 ng/mL	75.7–88.9	≤13.0	30
Vermicompost	SPE	HPLC-UV	5–50 µg/mL	0.67 µg/mL	–	–	31
Water	DLLME	CE-DAD	0.002–2 µg/mL	0.002 µg/mL	67.2–68.1	≤12.3	32
Food, vegetable	SPE	HPLC-UV	–	0.006 µg/g	–	–	GB/T23381-2009
Bean sprouts	FSPE	HPLC-UV	0.2–4.8 µg/mL	0.055 µg/mL	81.1-106.2	≤7.6	Present work

(DLLME: dispersive liquid–liquid microextraction; icELISA: indirect competitive enzyme-linked immunosorbent assay CE-DAD: capillary electrophoresis-diode array detection; GB/T: national standard of China)

FIGURE GRAPHICS

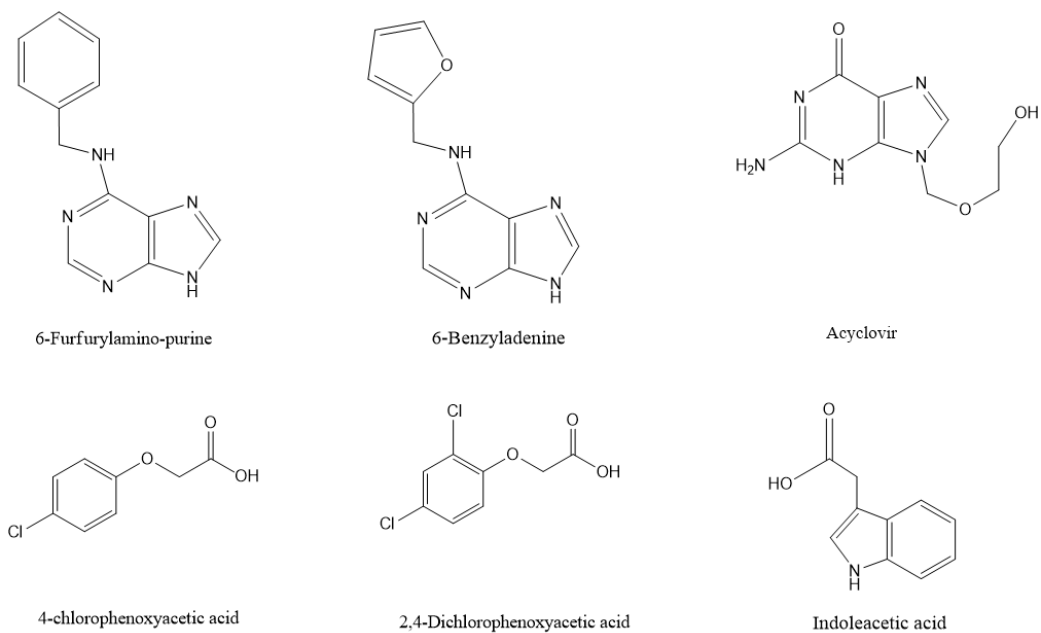


Figure 1

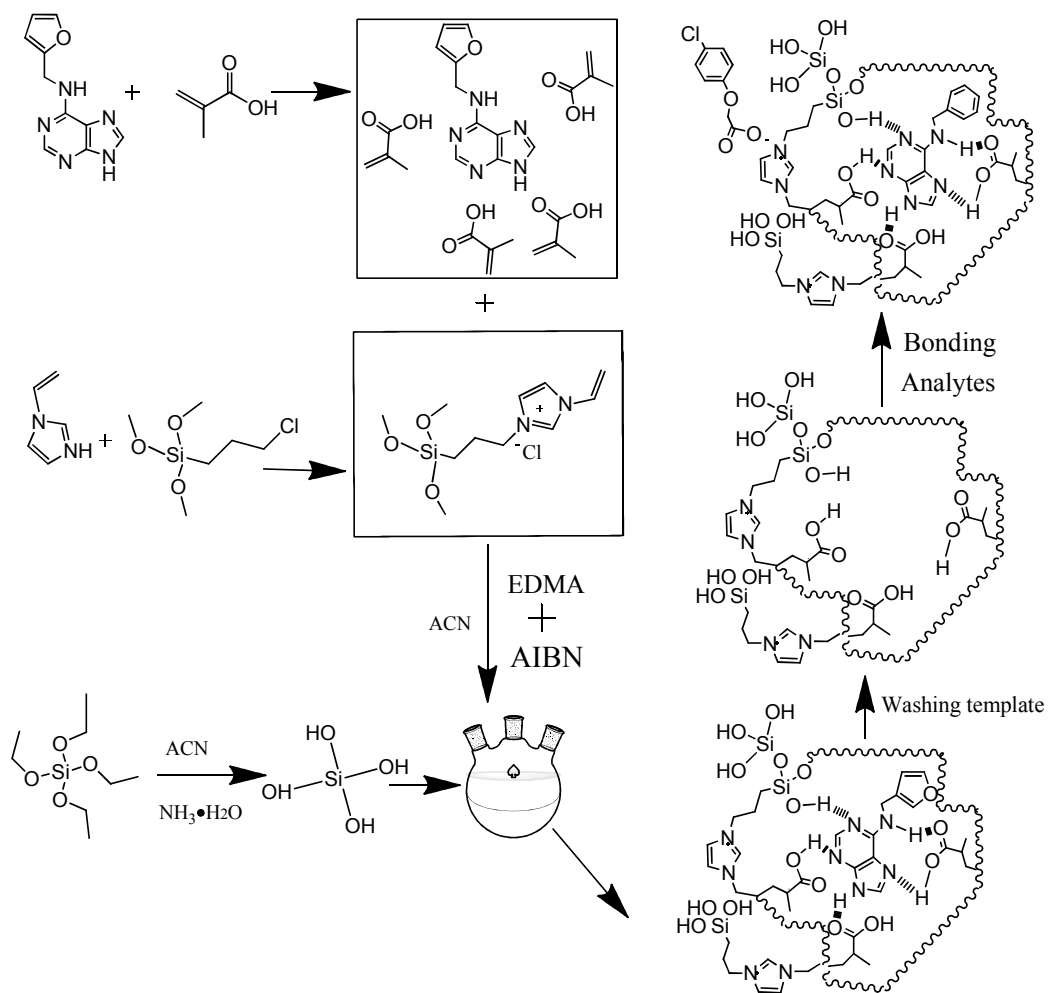


Figure 2

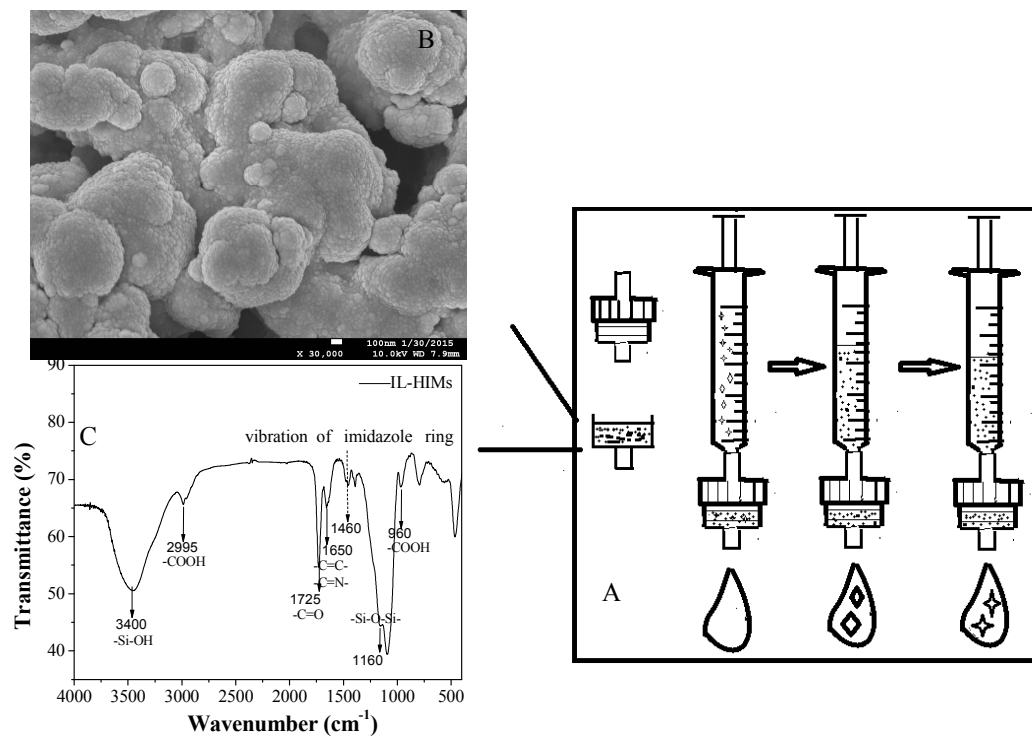


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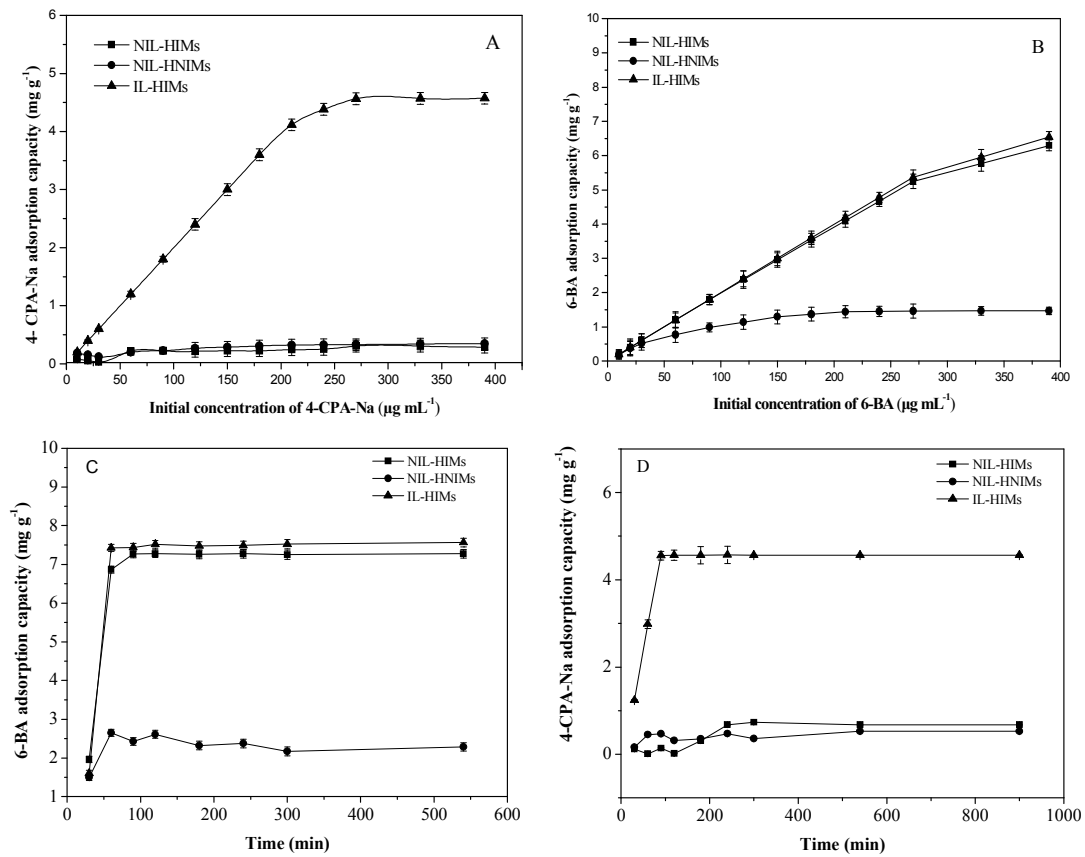


Figure 4

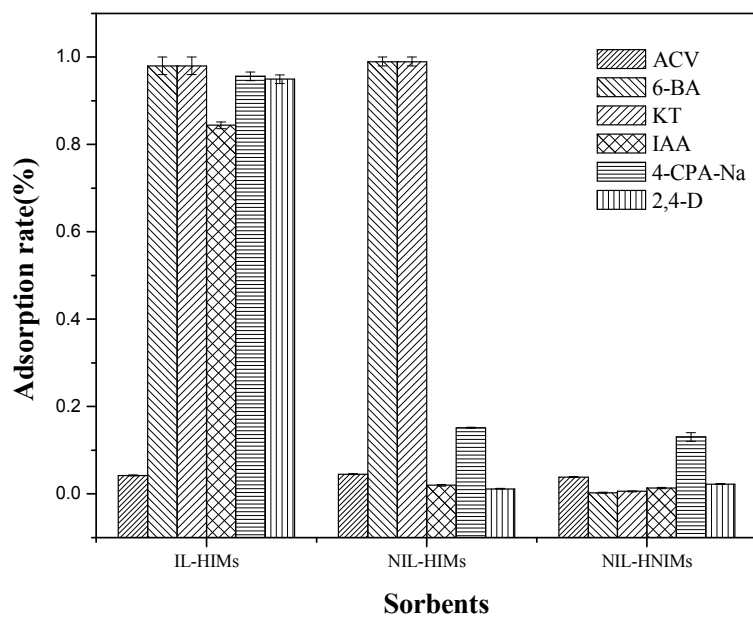


Figure 5

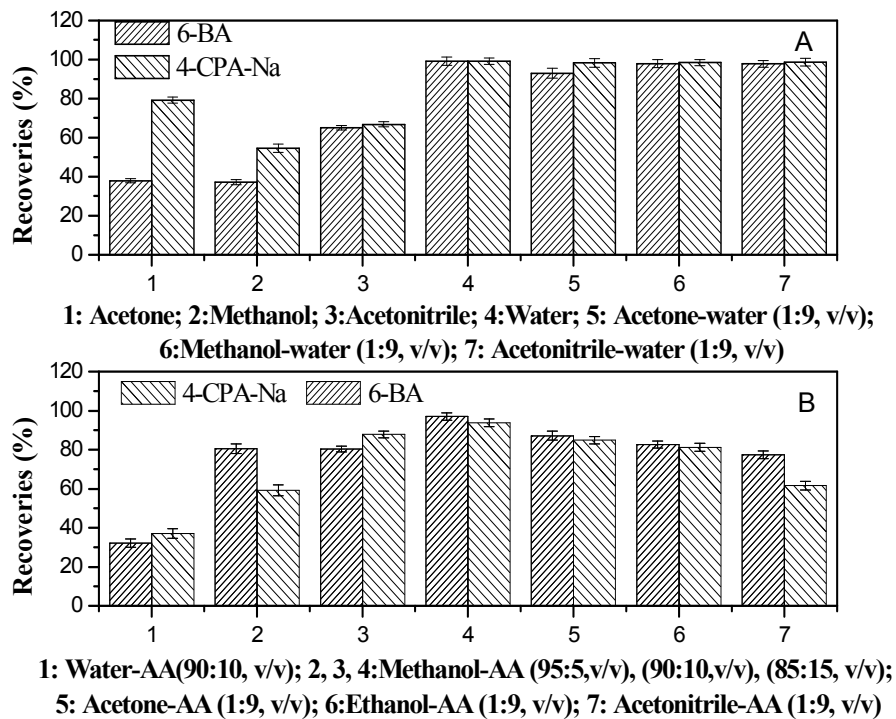


Figure 6

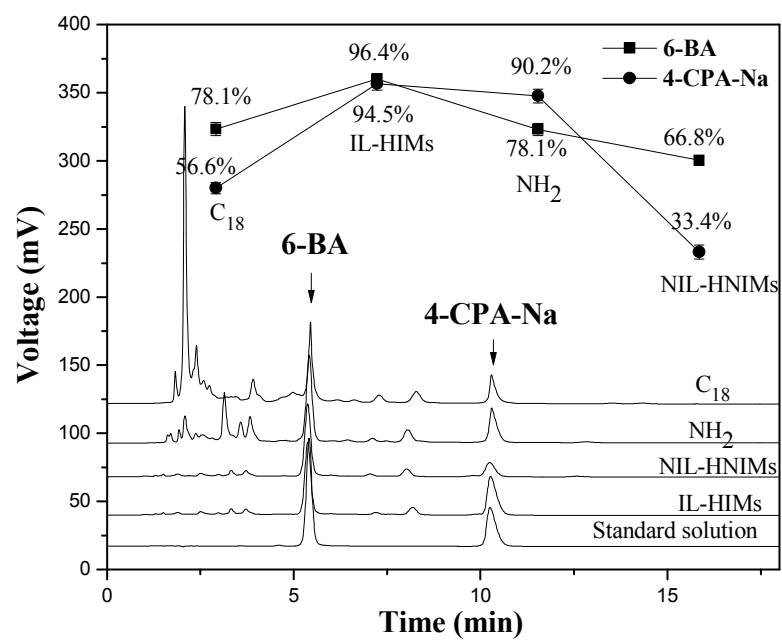


Figure 7

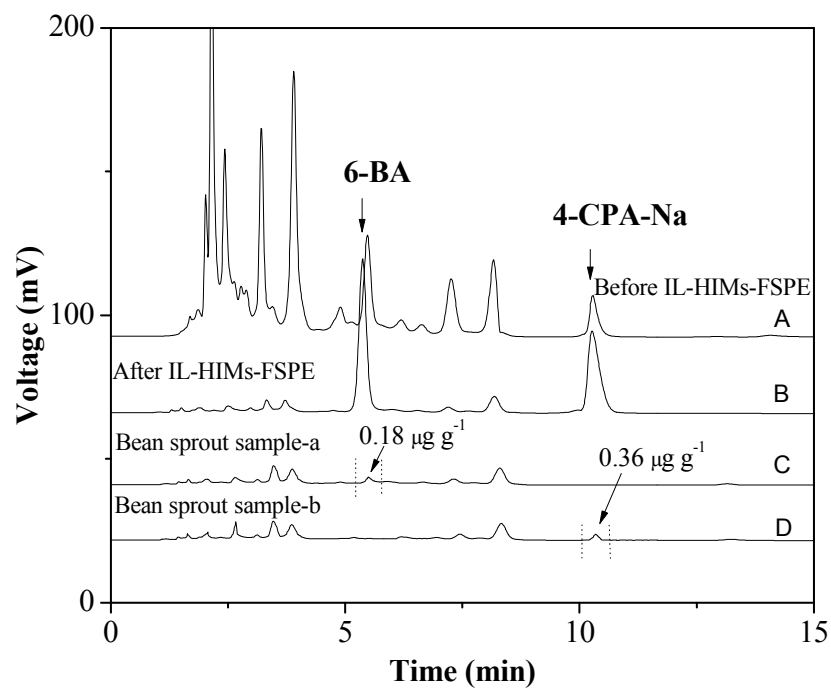


Figure 8

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