1   Deep eutectic solvents aqueous two-phase system based ultrasonically assisted

2   extraction of ursolic acid(UA) from *Cynomorium songaricum* Rupr.

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7   Abstract: Deep eutectic solvents(DESs) are new green solvents that have attracted the

8   attention of the scientific community mainly due to their unique properties and special

9   characteristics, which are different from those of traditional solvents.A method based

10   on   ultrasonically   assisted   deep   eutectic   solvent   aqueous   two-phase

11   systems(UAE-DES-ATPS) was developed for extracting ursolic acid (UA) from

12   *Cynomorium songaricum* Rupr. Four different types of choline chloride-based DESs

13   were   prepared.Choline   chloride-glucose   (ChCl-Glu)   exhibited   good   selective

14   extraction ability. An optimum DES-ATPS of 36% (w/w) ChCl-Glu and 25% (w/w)

15   K_2HPO_4 was considered to be a satisfactory system for extracting UA. Response

16   surface methodology (RSM) method was used to optimize the extraction of UA using

17   UAE-DES-ATPS. The optimum ultrasound-assisted conditions were as follows:

18   solvent to solid ratio of 15:1 (g/g), ultrasound power of 470 W, and extraction time of

19   54   min.   Compared   with   the   conventional   UAE   method,   the   yields   were

20   basically   the   same,   but   the   presented   method   had   higher   purity.   The

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21 structure of UA did not change between pure UA and UA in the upper phase by

22 UV–vis and FT-IR. This approach using ChCl-based DES-ATPS as

23 a novel extraction system and ultrasound as a source of energy provided better

24 choice for the separation of active components from other natural products.

25 Keywords: Ursolic acid, Choline chloride-Glucose, Aqueous two-phase systems,

26 Conventional UAE, Response surface methodology

27 1. Introduction

28   The stems of *Cynomorium songaricum* Rupr., known as SuoYang in China, are

29 mainly distributed in the Northwest and has been used in medical applications and

30 health care functions. Studies in the past have shown that SuoYang were

31 effective in improving enterocinesia, tonifying the kidneys, and relaxing bowel

32 function(Wang et al., 2016). *Cynomorium songaricum* Rupr. has many secondary

33 metabolites, such as organic acid, flavonoids, vitamins, polysaccharides, tannins and

34 triterpenoids(Jin et al., 2014).

35   Ursolic acid (UA) is the major active component among the triterpenoids. A large

36 number of experimental studies have shown a variety of pharmacological activities

37 have been found, such as antitumour activity(Oprean et al., 2016), antibacterial

38 activity(Srinivasan et al., 2017), antioxidant activity(Dzoyem et al., 2017),

39 macrophage autophagy(Podder et al., 2015), and attenuation of atherogenesis(Leng et

40 al., 2016).

41   Because of the good biological activity, separation of UA from *Cynomorium*

42 *songaricum* Rupr.has received more attention. Many methods, such as organic solvent

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43 extraction(Chen et al., 2014; Baranauskaitė et al., 2016), ethanol/non-aqueous solvent

44 by direct-heating extraction(Fan et al., 2016),ultrasound-assisted extraction (UAE)

45 (Chen et al., 2007), microwave-assisted extraction (MAE)(Fernandez-Pastor et al.,

46 2017;Verma et al., 2016; Zhong et al., 2016), enzymatic extraction (EE)(Zhou et al.,

47 2017), and subcritical water extraction (SWE)(Mlyuka et al., 2016)have been used to

48 extract UA from natural products. However,these techniques have disadvantages of

49 large amounts of organic solvents, long extraction times, and high energy

50 consumption. Meanwhile, aqueous two-phase systems (ATPS) ,as

51 a highly efficient and mild bioseparation technique, are composed of two kinds of

52 immiscibility polymers or one polymer and salts(Falcon-Millan et al., 2017; Murari et

53 al., 2017).When the concentration of chemical reagents reaches a certain value, two

54 incompatible phases are formed. Extraction techniques with ATPS

55 have been widely used in the separation and purification of

56 biomolecules(Falcon-Millan et al., 2017; Murari et al., 2017; de Barroset al., 2016;

57 Rahimpour et al., 2016).

58   The first time Bridges reported an ionic liquids (ILs)-based

59 ATPS was in 2003(Bridges et al., 2007).ILs have a wide liquid temperature range,

60 non-volatility, non-flammability, strong conductivity, high heat capacity,negligible

61 vapour pressure and good stability and are environmentally friendly(Gardas and

62 Coutinho,2008; Liu and Chen, 2006). An ILs-ATPS is mainly composed of an ionic

63 liquid and a salt in a certain concentration range, which

64 combines the advantages of ILs and ATPS[24,25](Borges et al., 2016; Shu et al.,

65   2016), is biocompatible, and has low steam pressure and good chemical stability and

66   dissolving properties(Sivapragasam et al.,2016). Based on these factors, ILs-ATPS has

67   been applied to the separation of biomolecules. Han et al. established preconcentrated

68   chloramphenicol (CAP) in a water-based on IL-ATPS(Han et al.,2011). Wang et al.

69   (2016) developed an IL-ATPS for the extraction of aconitum alkaloids. Zhang et

70   al.(2016) studied the extraction of UA from *Cynomorium songaricum* Rupr. by a

71   [Bmim]Cl/phosphates aqueous two-phase system. However, it was limited by

72   difficulties with synthesis and a high price. Moreover, imidazolium and pyridinium

73   ILs have certain toxicity(Docherty et al.,2005; Latała et al.,2009).The above

74   deficiencies have limited the wider deployment of many applications, such as in the

75   food and pharmaceuticals industries.

76   To surmount these faults, the emergence of a number of deep eutectic solvents

77   (DESs) that not only have inherited the advantages of ionic liquids but also have some

78   distinguishing features, including ease of preparation by mixing, lower cost,

79   and value in industrial application, has occurred (Dai et al.,2013; Bosiljkov et

80   al.,2017;Smith et al.,2014). A DES is a mixture of two or three components that are

81   composed of a hydrogen bond acceptor (such as choline chloride or betaine) and a

82   H-bond donor (e.g., amide, carboxylic acid and polyol). Choline chloride (ChCl)

83   is a kind of quaternary ammonium salt that is relatively cheap. ChCl-DESs are

84   attracting increasing attention, such as for   hydrolysis reactions(Lindberg et al.,2010),

85   removal of trace carbon dioxide(Li et al.,2008), and electroanalysis[37](Jhong et

4

86   al.,2009).Zeng et al(2014) and Xu et al(2015) developed as system for the extraction

87   of protein by a choline chloride-based DES-ATPS.

88   UAE is more effective with the advantages of a short extraction time, a higher yield

89   and a reduced solvent requirement(Xu et al.,2017;Tiwari, 2015). Ultrasound-assisted

90   deep eutectic solvent aqueous two-phase system extraction(UAE-DES-ATPS)

91   combines the advantages of both UAE and DES-ATPS, incorporates the less solvent

92   amount and has simultaneous extraction and separation of target ingredients to

93   remove impurities in the two-phase system(Smith et al., 2014).UAE-DES-ATPS is a

94   potential method for the extraction of active constituents.

95   In this text, the object of the present work was to establish an innovative, efficient,

96   and green method for extracting and separating UA from *Cynomorium songaricum*

97   Rupr. by UAE-DES-ATPS extraction. Target UA were changed from herb powders to

98   the top phase, and impurities remained in the bottom phase. To obtain a good aqueous

99   two-phase system, the DES type, DES, and salt concentration were studied. To

100   optimize UAE-DES-ATPS conditions, the extraction conditions (solvent to solid ratio,

101   ultrasound power, and extraction time) were optimized by response surface

102   methodology (RSM). This method was compared with conventional UAE using

103   ethanol as the extraction solvent. The extraction mechanisms were investigated by

104   FT-IR and UV-vis. The comparative analysis between the purity of UA in the

105   UAE-DES-ATPS and UAE using ethanol experiments were carried out by HPLC.

106   2. Experimental Section

107   2.1. Materials

108   The stems of *Cynomorium songaricum* Rupr. were provided by the Kaiyuan

109   Bio-Tech Development Center, Gansu Province, China, in November 2016. The

110   drying fresh samples were pulverized by a grinder and passed through a 30-mesh

111   sieve, and stored in vascula and collection bags. UA standards ($\geq$99%) were supplied

112   by Shanghai Yuanye Biotechnology Limited in China. Choline chloride (ChCl)

113   ($\geq$98%), glycerol ($\geq$99%), urea ($\geq$99%), ethylene glycol ($\geq$99%), and glucose ($\geq$99%)

114   were purchased from Shanghai Sinophram Chemical Reagent Co., Ltd,China.

115   2.2. Preparation of DESs

116   Different kinds of DESs were prepared by mixing up choline chloride with a

117   H-bond donor at 80 ℃ until clear solutions were formed. The molar ratio of choline

118   chloride to H-bond donor(HBD) (glycerol, urea, glucose, and ethylene glycol) can be

119   seen in table 1. The preparation of four DESs was characterized by FT-IR, as

120   shown in Fig. 1. Broad and strong peaks at the wavenumbers between 3342 and

121   3329 cm^{-1} were assigned to the OH stretching bands at the wavenumber of

122   3300 cm^{-1}(Abo-Hamad et al., 2015),which indicated the presence of lots of hydrogen

123   bonds in four DESs.

124   2.3. Preparation methods for phase diagrams

125   Cloud point is a very effective method to produce the phase diagrams (Li et al.,

126   2005). A certain amount of DESs was added to a test tube, and

127   a specific salt was added to the test tube until the turbidity appeared.

128   After the addition of water, the solution become clear.This process was repeated

129   until a binodal curve was obtained.

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130   2.4 Preparation of crude UA extract

131   UA was extracted from *Cynomorium songaricum* Rupr.(50 g) by using 85%

132   ethanol(Wei et al., 2014)(1000 mL) for 2 hours at 70℃. The two extracts were

133   separated by centrifuge, and clear supernatant extracts were concentrated by

134   decompression, and dried to produce the crude UA extract(5.15 g).

135   2.5 DES-ATPS selection: preliminary studies

136   To search for an optimal DES-ATPS that offered a high extraction ratio, the crude

137   UA extract prepared in Section 2.4 was used instead of sample powders to evaluate

138   various DES-ATPS without the use of ultrasound. A few grams of salt and crude UA

139   extract were added to deionized water, and different DESs were added . The mixtures

140   were mixed by a vortex stirrer for 5 min at 22 ± 1℃ and left to stand for 10 minutes,

141   and two phases were formed. The volumes of the upper and bottom phases were

142   tested. The contents of UA in the upper and bottom phases were

143   determined. The function below can be utilized to calculate the partition coefficients

144   (K) and extraction ratio (E).

145    $K = \dfrac{C_t}{C_b}$

146    $E = \dfrac{C_t V_t}{C_t V_t + C_b V_b} \times 100\%$

147   where C_t and C_b represent the content of UA (μg/mL)in the upper and bottom

148   phases,which was determined by HPLC analysis,respectively, and V_t and V_b stand for

149   the upper and bottom phases volumes (mL), respectively.

150   2.6 UAE-DES-ATPS to extract UA from *Cynomorium* powders

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151   In the extraction process,the optimal ATPS of 36% (w/w) ChCl-Glu-based DES and

152   25% (w/w) K_2HPO_4 was chosen as based on partition coefficients (K) and

153   extraction ratio (E) K_2HPO_4 and deionized water were mixed in centrifuge tubes and

154   vortexed. Then, the ChCl-Glu-based DES was added with sufficient mixing and

155   allowed to separate into phases. The drying *Cynomorium* powders were extracted by

156   DES-ATPS. UAE-DES-ATPS was designed with a specific solvent to solid ratio,

157   ultrasound power, and extraction time. After each extraction was over, the extract was

158   filtrated by a filter paper to remove the *Cynomorium* residue.Two phases were formed

159   by leaving them stand for 10 min. Most of the impurities were presented in the

160   salt-rich bottom phase.The UA was mainly transferred to the DES-rich upper phase,

161   which was determined by HPLC analysis.The yield of UA was calculated using the

162   following formula:

163    $$\text{Yield(mg/g)} = \frac{C_t V_t}{1000 M_1}$$

164   where C_t and V_t are the UA concentration (μg/mL) and upper phase volume (mL)

165   respectively. M_1 is the *Cynomorium* powders quality in the ATPS(g).

166   The upper phase was separated from ATPS by a separation funnel, underwent solid

167   phase extraction (SPE),and was concentrated and dried in a vacuum. The current

168   formula for the purity of UA was as follows.

169    $$\text{Purity} = \frac{C_t V_t}{10^6 M_0} \times 100\%$$

170   where C_t and V_t are the UA concentration (μg/mL) and upper phase volume

171   (mL),respectively.M_0 is the quantity of upper phase concentrated and dried in vacuum

172   (g).

173   2.7 Experimental design

174   On the basis of preliminary studies, the experimental parameters, including the

175   solvent to solid ratio, ultrasound power, and extraction time, were optimized with a

176   single factor experimental design. According to the above experimental results, the

177   RSM was used to optimize the UAE-DES-ATPS extraction conditions. The

178   effects of the solvent to solid ratio, ultrasound power, and extraction time were

179   investigated with a Box-Behnken design (BBD). The variables and levels are

180   presented in Table 2. The response obtained from the BBD was estimated by the

181   multiple regression coefficients of a second order polynomial, which was as follows.

182    $Y_m = \beta_0 + \sum \beta_m X_m + \sum \beta_{mm} X_m^2 + \sum \beta_{mn} X_m X_n$

183   where Y_m represents the corresponding value, and $X_m X_n$ are independent variables

184   that affect the dependent variable Y. β_0 is a fixed value, and B_m, B_{mm}, and B_{mn}

185   represent the mth linear factor, the mmth second-order coefficient , and the mnth

186   cross-correlation coefficient, respectively.

187   2.8 HPLC analysis

188   The contents of UA in the DES-rich upper phase was determined by HPLC analysis.

189   An Agilent HC-C18 (4.6 mm $\times$ 150 mm) column was used. The mobile phase was

190   methanol-water (50:50). The current velocity was 0.5 mL/min. The wavelength of

191   detection was 210 nm. The contents of the UA was determined using the

192   normalization method in the effective concentration range of 130-528 µg/mL. The

193   linear equations for peak area and UA concentration were Y=39.197x-850.99,

194   (R^2=0.9977).

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195   2.9 Statistical analysis

196   All the experiments were repeated three times. Origin version 8.0 and

197   Design-Expert 7.1.6 were used to analyse the data, which are expressed as the mean

198   ± SD.

199   3. Results and discussion

200   3.1 Phase diagrams of the selected DES-ATPS

201   Three types of salts (K_2HPO_4, KH_2PO_4,and $(NH_4)_2SO_4$) were used to generate

202   DES-ATPS.The phase-forming ability of different salts was determined by the Gibbs

203   energy of hydration of ions(-2379, -1650 and -760kJ/mol for K_2HPO_4, $(NH_4)_2SO_4$,

204   and KH_2PO_4, respectively)(Marcus , 1987), which was in accordance with the phase

205   separation ability.Only K_2HPO_4 can form two phases. The phase diagram data was

206   prepared by cloud point methods at 22 ± 1°C. As shown in Fig. 2, The order of the

207   phase   forming   ability   of   different   DESs   and K_2HPO_4

208   were:ChCl-U>ChCl-Glu>ChCl-G>ChCl-EG.ChCl-U has better phase forming ability

209   in ATPS than others, which was consistent with results reported in the literature(Farias

210   et al., 2017). This phenomenon may have occurred because water is one of the

211   dominant factors affecting DES integrity,which is destroyed in DES-ATPS by

212   breaking the hydrogen bonds within the HBD:HBA complex(Passos et al., 2016).The

213   formation of ATPS was regulated by the ChCl, with the HBD playing a minor but

214   important role during the formation of the phase.Different DESs have different

215   properties such as viscosity, density, and hydrophilicity.Hydrophilicity may be

216   one of the important factors   that causes the phase formation.In general,

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217　　binodal curves remain close to the point of intersection of coordinate axes, and the

218　　hydrophilicity of DESs was on the decline with a small amount of salt to promote

219　　the phase formation.

220　　3.2 The selection of the DES-ATPS

221　　　　Based on the results of the phase diagrams, extraction and separation of UA by

222　　different DESs/K_2HPO_4 aqueous two-phase systems were compared. The UA was

223　　mainly transferred to the DES-rich upper phase, and little of the UA was presented in

224　　the salt-rich bottom phase.The results are shown in Fig. 3a. ChCl-Glu/K_2HPO_4-ATPS

225　　had a higher the partition coefficient (K) and extraction efficiency than other

226　　ChCl-based ATPS.The extraction of UA from *Cynomorium songaricum* Rupr.

227　　by ChCl-Glu/K_2HPO_4-ATPS was proposed in a follow-up study.

228　　 3.3 Effects of ChCl-Glu mass fraction on DES-ATPS

229　　　To evaluate the effect of the ChCl-Glu mass fraction on the aqueous two-phase

230　　system, the range of values including 32-40% (w/w) ChCl-Glu and 25% (w/w)

231　　K_2HPO_4 were studied. Fig. 3b indicates that the extraction efficiency and the partition

232　　coefficient (K) increased over the ChCl-Glu mass fraction range of 32-36%, while the

233　　extraction efficiency and K decreased over the ChCl-Glu mass fraction range of

234　　36%-40%.This result can properly describe the size change of ChCl-Glu micelles in

235　　the upper phase(Li et al., 2016), and UA was prone to gathering around

236　　ChCl-Glu micelles, which was more advantageous for distribution in the upper

237　　DES-rich phase. However,with increasing mass fraction of ChCl-Glu, the upper phase

238　　increased the viscosity and weak mobility, which hindered UA from going to the

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239   upper phase. Therefore, 36% (w/w) ChCl-Glu was confirmed for the next step

240   experimental step.

241   3.4 Effects of the mass fraction of K_2HPO_4 on DES-ATPS

242   Based on the above, 36% (w/w) ChCl-Glu and 21%-29% (w/w) K_2HPO_4 ATPS were

243   investigated. The mass fraction of crude UA in ATPS was 1% (w/w). From Fig. 3c,

244   the extraction efficiency and K increased with the increase in the K_2HPO_4 mass

245   fraction from 21% (w/w)to 25% (w/w).However, the extraction efficiency and K

246   decreased with the further increase in the mass fraction of K_2HPO_4 from 25% (w/w)

247   to 29% (w/w).This phenomenon may be due to the reduced salting-out

248   effect.With increasing the K_2HPO_4 concentration, a great amount of K_2HPO_4 had an

249   effect on the existence of ChCl-Glu and UA in the bottom phase, which increased the

250   hydrophobicity of the bottom phase. The salt ions competed for water molecules with

251   UA, causing the salts to be soluble in the bottom phase. As a result, the ChCl-Glu-UA

252   complexes were transferred from the salt-rich bottom phase to the DES-rich

253   phase.High levels of K_2HPO_4 in ATPS can reduce the water   content   and increase

254   viscosity in the upper phase, and UA was barred from the upper phase. Therefore,

255   using 36% (w/w) ChCl-Glu and 25% (w/w) K_2HPO_4 in ATPS was selected for further

256   studies.

257   3.5. Optimization of extraction UA by UAE-DES-ATPS

258   3.5.1 Single factor experiment

259   An aqueous two-phase system of 36% (w/w) ChCl-Glu and 25% (w/w) K_2HPO_4

260   was selected to extract UA from *Cynomorium songaricum* Rupr. by UAE. The

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261 probable parameters influencing the extraction process were systematically studied in

262 a single factor analysis of the solvent to solid ratio, ultrasound power, and extraction

263 time. Of all these factors,when one factor was investigated, others factors were keep

264 constant.

265 The solvent to solid ratio is one of the most important factors that can influence the

266 UA extraction yield. Different ratios of solvent to solid were studied (7.5-37.5,

267 g/g)(see Fig. 4a).It was found that the yield of UA increased with increasing the

268 solvent to solid ratio from 7.5 g/g to 22.5 g/g, while the yield of UA decreased over

269 the solvent to solid ratio range of 22.5 g/g-37.5 g/g.These phenomenon can be explain

270 as follows:(1). DES-ATPS increased expansion of plant cell wall, which is beneficial

271 to increase the contact area of solvent and plant material.(2).A relatively high

272 solvent-solid ratio related to concentration difference between plant tissue of the host

273 and exterior solvent, can increase the driving force for mass transfer.However,the

274 solvent to solid ratio was too high, the distance of the diffusion of water into the

275 intra-cell of plant material was increased.Meanwhile,large amounts of impurities were

276 extracted, the effect of the mass transfer became worse.Thus, a solvent to solid ratio

277 of 15-30 g/g was consider for the next step optimization.

278 Ultrasound power was one of the chief parameters affecting extraction. Different

279 ultrasound powers including 100 W,200 W,300 W,400 W, 500 W and 600 W, were

280 investigated (see Fig. 4b).With the increase in ultrasound power, the yield of UA first

281 increased with ultrasound power up to 400 W and then decreased. The major reason

282 for the phenomenon was that the target compound might degrade at high ultrasound

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283   power, and the yield of UA may decrease. The results were the same as those in the

284   literature(Lou et al., 2010; Dey et al., 2013). Based on the above results, an ultrasound

285   power of 300-500 W was chosen to optimize the tests.

286   To a certain extent, the yield of UA was linked to the extraction time. As shown in

287   Fig. 4c, the yield of UA increased with the increase in extraction time.When the

288   extraction time was over 50 min, the yield of UA declined. Ultrasonic energy can

289   promote heat accumulation, the extracting rate of target product became

290   greater.However,the longer the extraction time and the higher the temperature, UA

291   structure was destroyed and affected the yield. For the aforementioned reasons, an

292   extraction time of 50 min was determined as central point of response surface design.

293   3.5.2 response surface method

294   Single factor analysis can only analyse the effects of one factor. For multiple

295   factors,we should also consider the interactive effect of influencing factors.

296   Accordingly,   to   optimize   the   ultrasonic   extraction   process,   an

297   aqueous two-phase system of 36% (w/w) ChCl-Glu and 25% (w/w) K_2HPO_4 was

298   selected to extract UA from *Cynomorium songaricum* Rupr.by UAE. The solvent to

299   solid ratio (A), ultrasound power (B), and extraction time (C) were identified as three

300   factors to optimize the UA extraction process. A Box-Behnken experimental

301   design with three factors and three levels was used as shown in table 2.

302   As shown in table 3, validation of model significance was performed with variance

303   analysis and F-tests. The regression equation was built according to variance analysis

304   of the regression model:

305     $Y=20.25+0.21A+1.92B-0.11C-1.35AB-2.84AC-0.24BC-0.72A^2-2.28B^2-2.80C^2$

306   The larger the F-value is, the more meaningful the model. The model F-value of

307   18.32 showed that the model was effective. The model F-value had a 0.05% chance of

308   occurring because of the attending of noise. If "Prob>F" was smaller than 0.05, the

309   model terms were significant. The independent variable of B, the interaction terms of

310   AB and AC, and the quadratic terms of B^2 and C^2 were significant model terms. The

311   lack of fit F-value (p value=0.46)was not significant relative to the pure error. The

312   lack of Fit F-value had a 72.26% chance of occurring due to noise. The R-squared and

313   adjusted R-squared were 0.9593 and 0.9069, respectively, which indicated that the

314   independent variables and the dependent variables had linear relations.Adequate

315   precision represented the signal-to-interference ratio (SIR). The SIR of 10.479

316   indicated a suitable signal.

317   Fig. 5 presents the interaction between the solvent to solid ratio (A), ultrasound

318   power (B), and extraction time (C). Fig. 5a showed that the yield of UA increased as

319   the solvent to solid ratio increased when the ultrasound power was kept constant.

320   Similarly, increasing the ultrasound power obviously improved the yield of UA. As

321   shown in Fig. 5b, when the solvent to solid ratio and extraction time increased, the

322   yield of UA also increased. From Fig. 5c, the yield of UA increased with a

323   small extraction time difference. With the increase in extraction time, the yield of UA

324   decreased. Small quantities of UA molecular structure were damaged

325   when extending the extraction time alone. Thermal effects and mechanical effects

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326   caused by ultrasonic cavitation, which destroys the molecular bonds of water and

327   forms free radicals, may be a main cause for structural destruction.

328   Based on this model, the prediction values of UA was 21.07 mg/g under the

329   following extracting conditions: 15:1 for the solvent to solid ratio, 470 W for the

330   ultrasound power, and 54 min for the extraction time. Comparing the values predicted

331   by the model with the experimental result, the yield of UA was 21.15 mg/g according

332   to real experiments, which was consistent with prediction values, and would provide

333   an effective method to extract UA from *Cynomorium songaricum* Rupr. based on

334   UAE-DES-ATPS.

335   3.6 Comparison of UAE-DES-ATPS and conventional UAE

336   To establish a green, effective extraction method, the UAE-DES-ATPS method was

337   compared with the traditional UAE method. With an ATPS of 36% (w/w) ChCl-Glu

338   and 25% (w/w) K_2HPO_4, the optimal extraction conditions were 15:1 for solvent to

339   solid ratio, 470 W for the ultrasound power, and 54 min for the extraction time.

340   The experimental results (Table 4) indicated that the traditional UAE method

341   produced more extract. The yields of UA between UAE-DES-ATPS and conventional

342   UAE were largely the same. UAE-DES-ATPS and conventional UAE extract

343   chromatograms are shown in Fig. 6. Highly purified UA from *Cynomorium*

344   *songaricum* Rupr. could be obtained by UAE-DES-ATPS, which presented a greater

345   purification capacity.

346   3.7 The analysis of extraction behaviour for UAE-DES-ATPS

347   3.7.1 FT-IR spectra

348   FT-IR spectra can provide an effective way to analyse the structures of

349   compounds. As shown in Figure 7, it can be seen from FT-IR spectra that the main

350   characteristic peaks positions were almost the same between pure UA and UA in the

351   DES-rich phase. The results showed that the structure of UA remain unchanged

352   during the extraction process.

353   3.7.2 UV–vis

354   UA in ethanol and in the DES-rich phase after extraction were studied with the

355   UV-vis spectral method. As shown in Fig. 8, UA in the two solvents had the same

356   shape curve and maximum absorbance peaks at 210 nm.The results showed that the

357   DES could not form new chemical bonds with UA.

358   4. Conclusions

359   In the experiment, UA was prepared from *Cynomorium songaricum* Rupr. by

360   UAE-DES-ATPS. According to the types of DES-ATPS studied, DES-ATPS was

361   composed of ChCl-Glu and K_2HPO_4. Based on that finding, the effects of the solvent

362   to   solid   ratio,   ultrasound   power,   and   extraction   time   on   the

363   extraction of UA from *Cynomorium songaricum* Rupr. were studied by

364   response surface methodology. A novel and effective method for the extraction of UA

365   based on UAE-DES-ATPS was developed. In the ATPS of 36% (w/w) ChCl-Glu and

366   25% (w/w) K_2HPO_4, the optimal extraction conditions included 15:1 for the solvent to

367   solid ratio, 470 W for the ultrasound power, and 54 min for the extraction time. With

368   these conditions, the yield of UA was 21.10 mg/g. UV–vis and FT-IR results showed

369   that the molecular structure of UA had not changed compared with standard UA.

370   Recycling the DES is one of the most important problems in DES-ATPS.Much

371   literature has focused on the extraction of natural products and enzyme catalysis but

372   has overlooked the recycling of the DES.Jeong(2015) and co-workers had recycled

373   the DES by freeze-drying the extract after recovering it, which provided a useful

374   reference for a follow-up study.

375   The stability of DESs in ATPS should be considered. According to Farias et

376   al.(2017)and Passos et al.(2016), ChCl played a major role in controlling the

377   construction of ATPS, while the HBD had only a small effect. The hydrogen bonds of

378   the DES were destroyed in the prepared ATPS. In addition, DESs were prepared by a

379   heating method, and a higher temperature may lead to the degradation of the heat

380   sensitive component parts such as amino acids. ChCl was reacted by Hofmann

381   elimination in the presence of an alkali. The next step is for us to explore the extract

382   kinetics and extract mechanisms for extracting UA by UAE-DES-ATPS.

383   Acknowledgement

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548   **Captions for Tables**

549   Table 1

550   The solvent composition of the four deep eutectic solvents prepared

551

552   Table 2.

553   Results of the three-factor and three-level response surface design

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555   Table 3

556   Analysis of the variance (ANOVA) for the square response surface model.

557

558   Table 4

559   Comparison between UAE-DES-ATPS and conventional UAE used for the UA yield

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561

562   **Captions to Figures**

563   Fig. 1   FT-IR spectra of four different DESs.

564

565   Fig. 2   Phase diagrams of four different DESs/salt aqueous two-phase systems

566   Fig. 3 Effect of different factors (different (a) DESs-ATPS; (b) ChCl-Glu mass fraction; (c)

567   K_2HPO_4 mass fraction) on the partition coefficient (K) and extraction efficiency(E).

568

569   Fig.4. Effect of the parameters of UAE-based DES-ATPS on the yield of UA: (a)solvent to solid

570   ratio (conditions:ultrasound power was 400W, extraction time was 50 min), (b)ultrasound

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571 power (conditions: solvent to solid ratio was 22.5 g/g, extraction time was 50 min), and (c)

572 extraction time (conditions: solvent to solid ratio was 22.5 g/g,ultrasound power was 400

573 W).

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575 Fig. 5 Response surface curves for the extraction of UA from *Cynomorium songaricum* Rupr.

576 showing the interaction between different factors.(a) solvent to solid ratio (g/g)and

577 ultrasound power(w),(b)solvent to solid ratio (g/g) and extraction time (min), (c)

578 ultrasound power(w) and extraction time (min).

579

580 Fig. 6 HPLC chromatograms for the samples after UAE-DES-ATPS (a) and UAE-ethanol(b).

581

582 Fig. 7 FT-IR spectra of pure UA (a), UA in the DES-rich phase (b), and pure ChCl-Glu (c).

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584 Fig. 8 UV-vis spectra of UA in ethanol and in the DES-rich phase after extraction

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595   Fig.1

ChCl-G

ChCl-Glu

ChCl-U

ChCl-EG

wavenumber(cm^{-1})
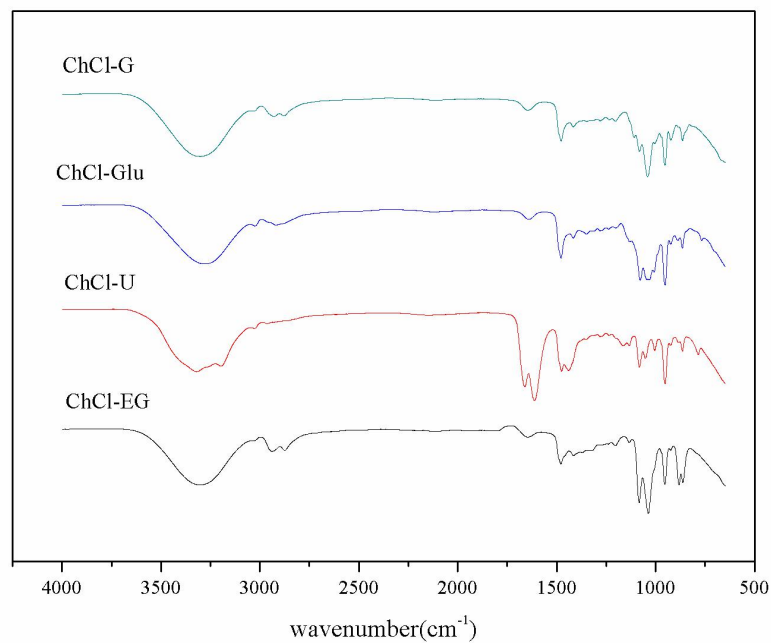
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620   Fig.2
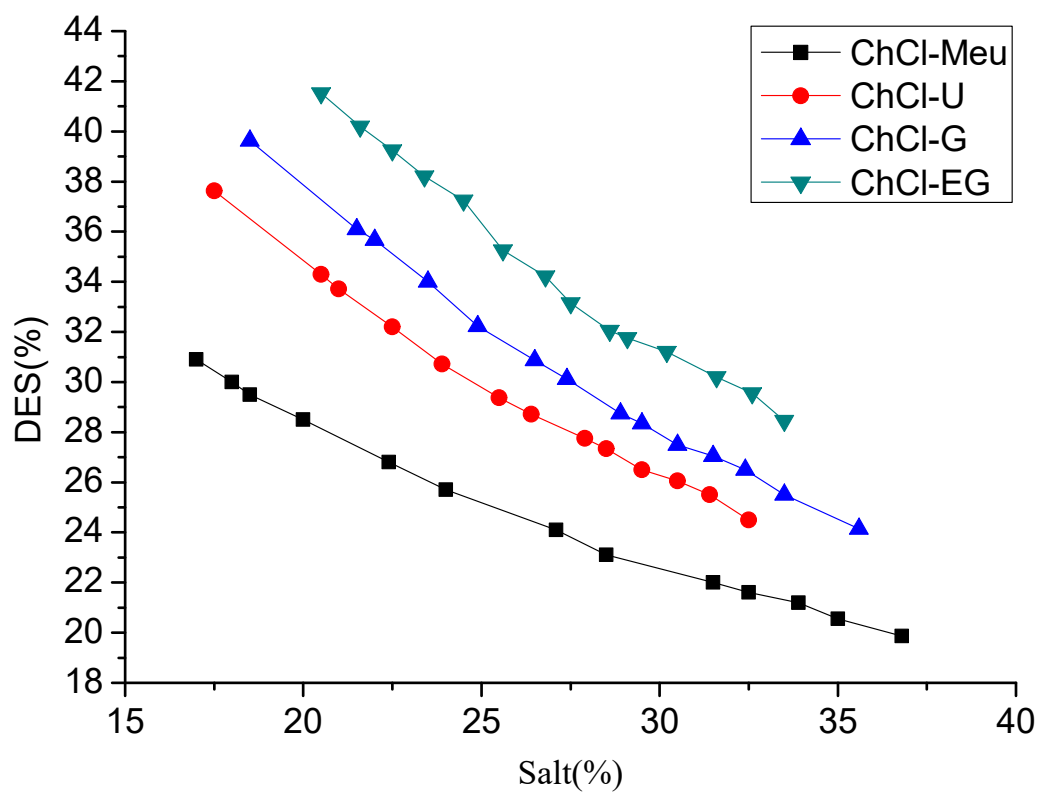
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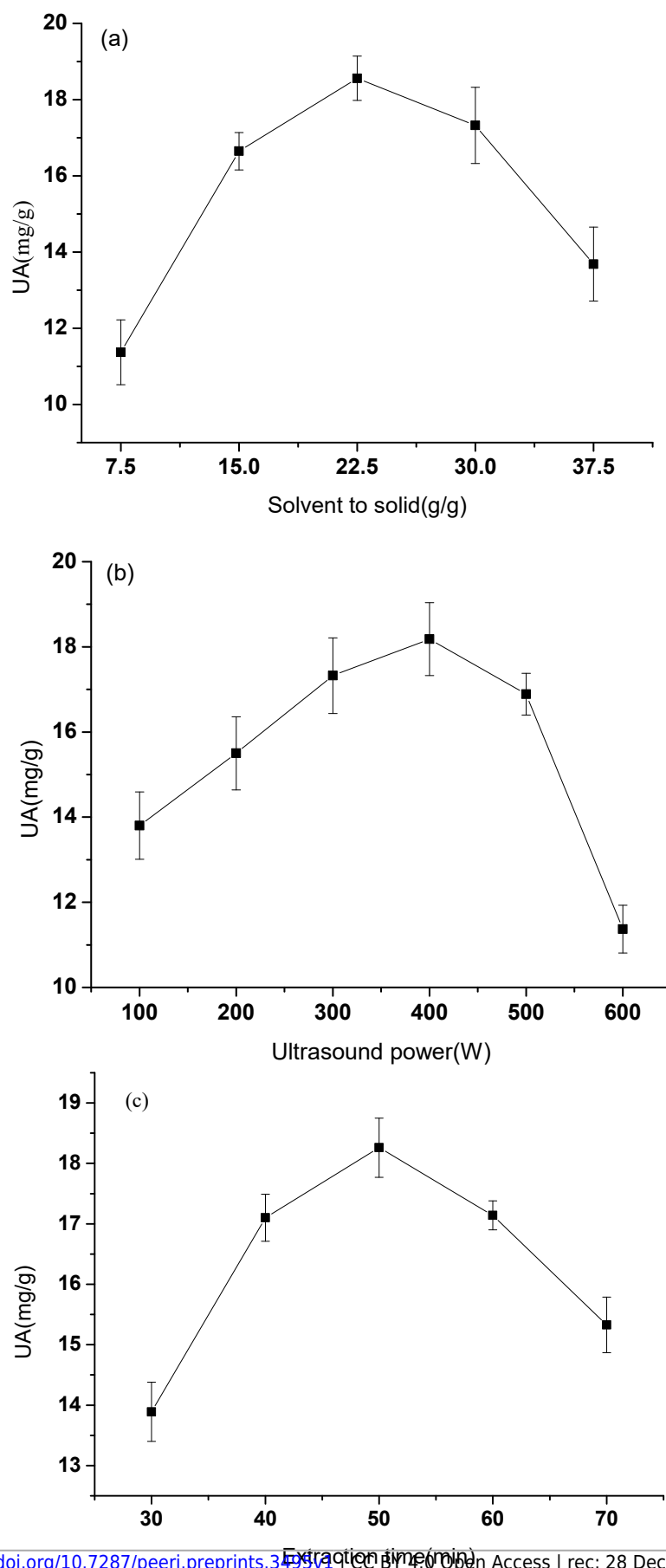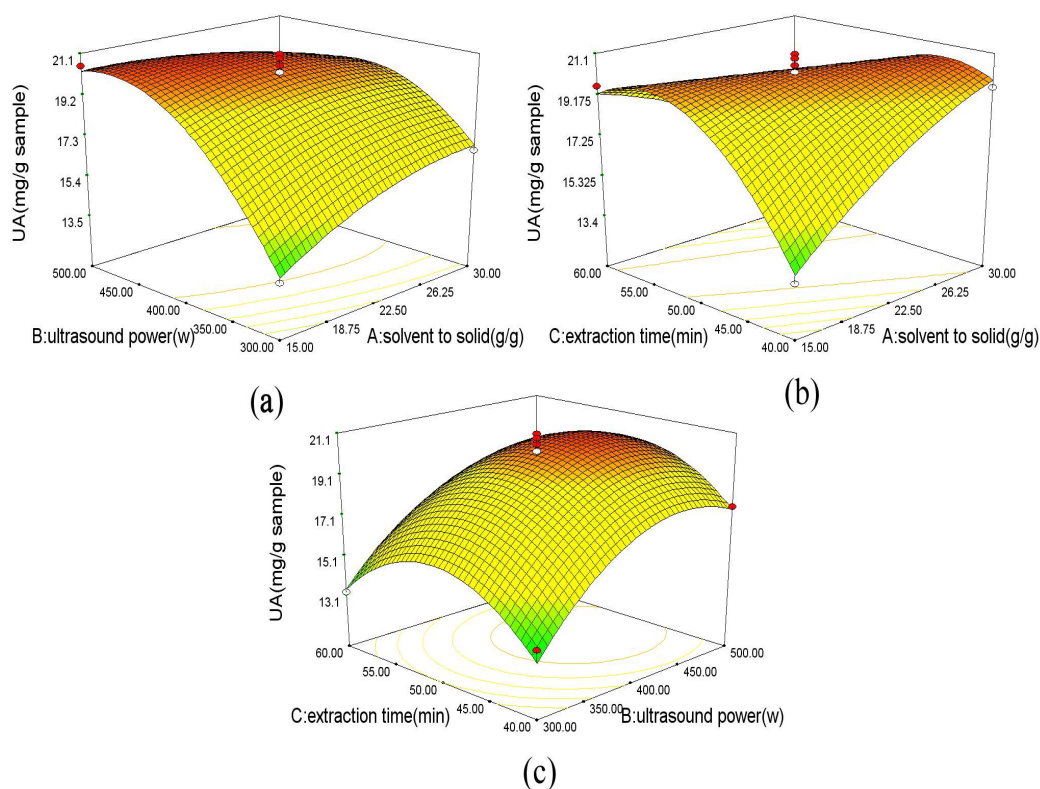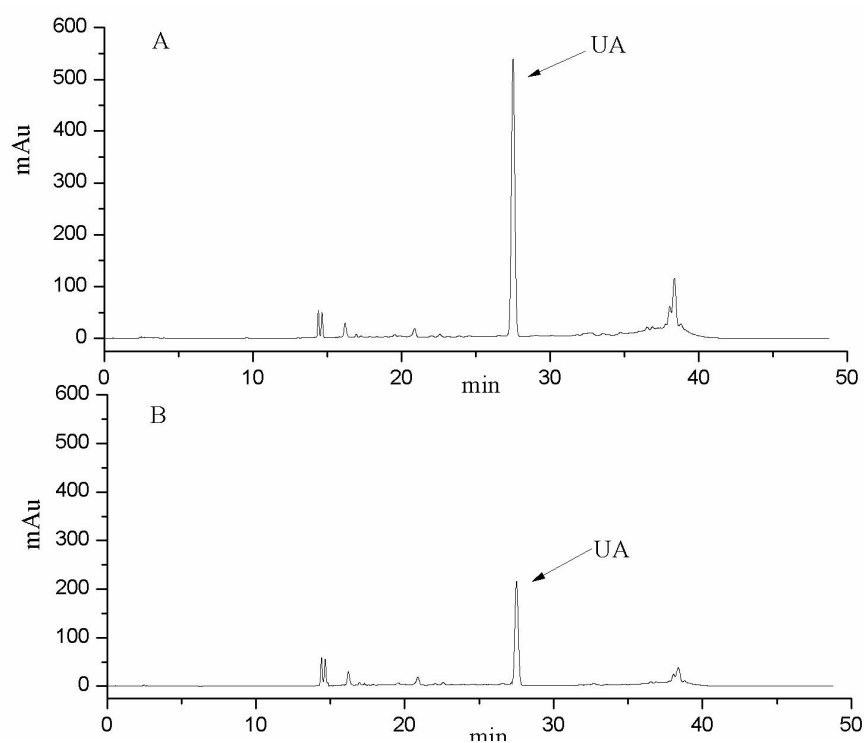637
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642   Fig.3

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684   Fig.4

(a)

UA(mg/g)

Solvent to solid(g/g)

(b)

UA(mg/g)

Ultrasound power(W)

(c)

UA(mg/g)

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Extraction time(min)

724   Fig.5

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(a)

(b)

(c)

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736   Fig.6

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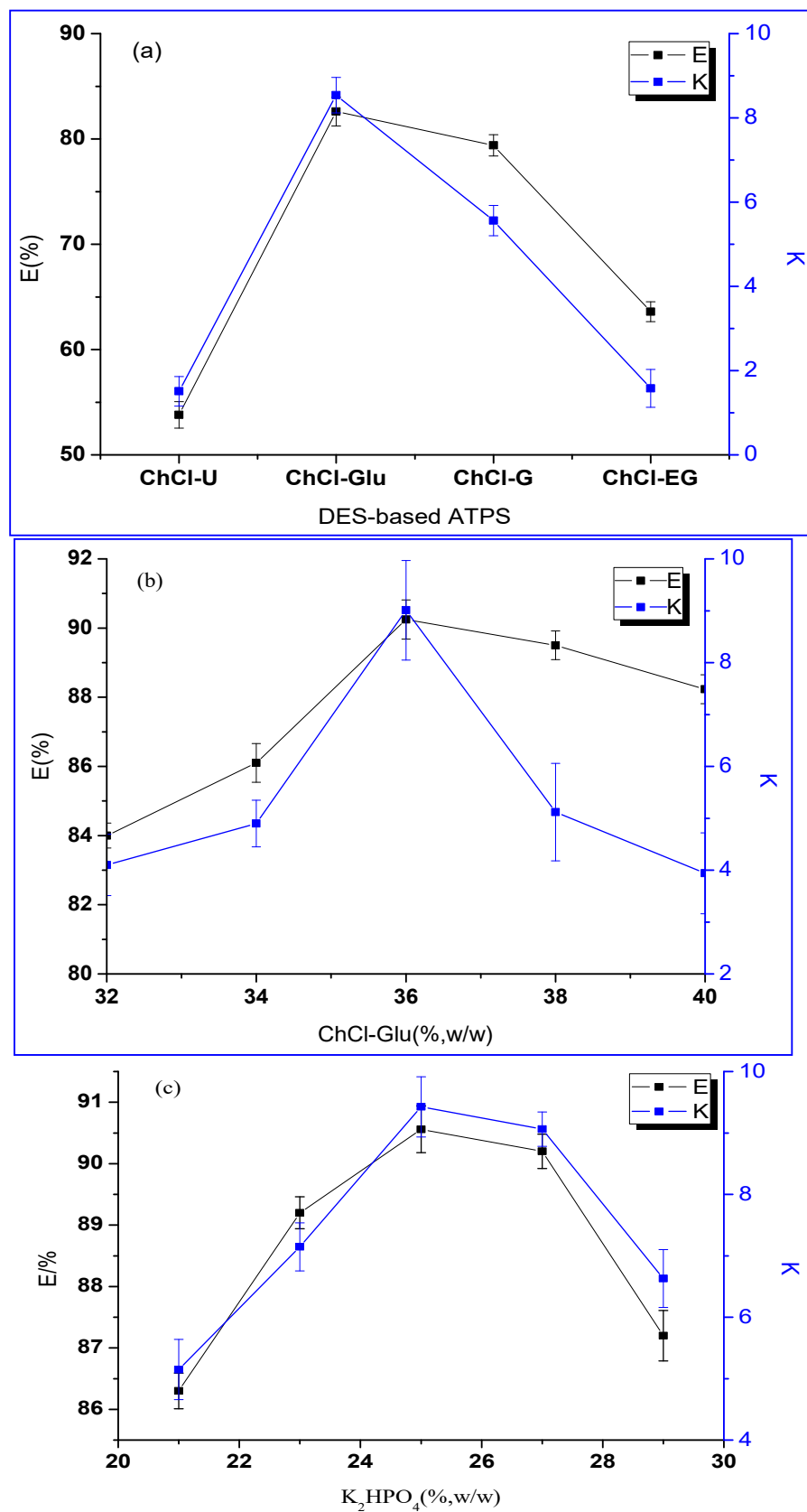
750   Fig.7
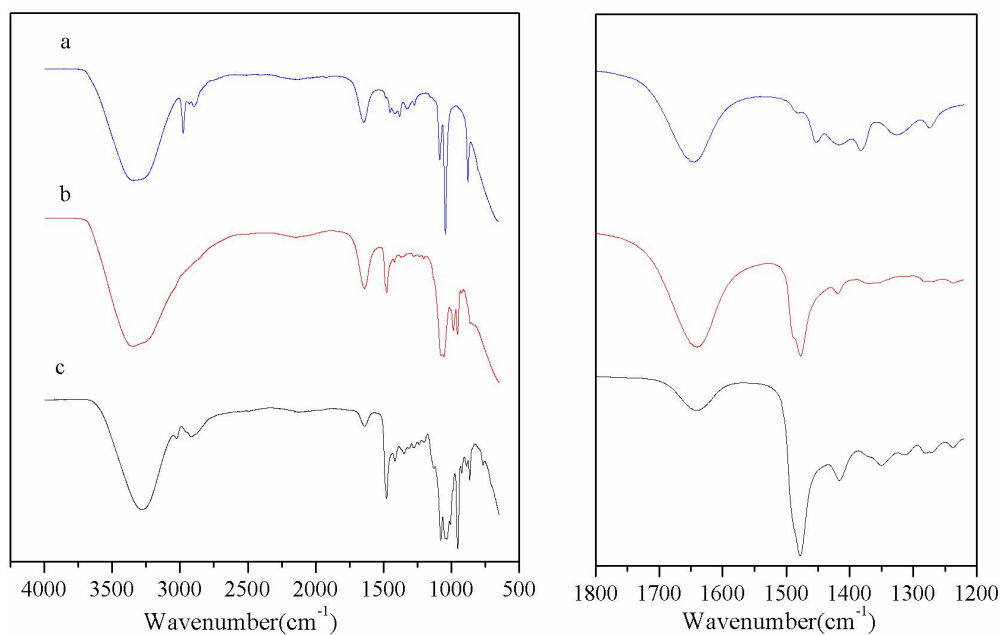
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764   Fig.8
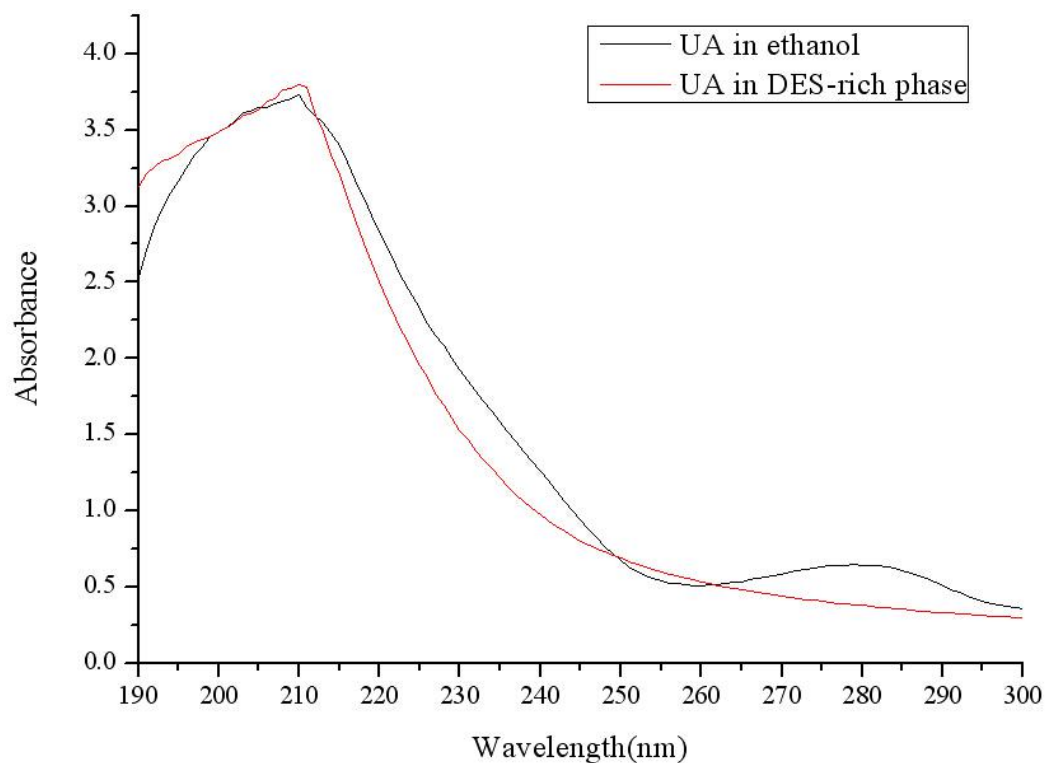
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Table 1. The solvent composition of the four deep eutectic solvents prepared

Quaternary ammonium salt	Hydrogen-bond donors (HBD)	Molar ratio of salt to HBD	Abbreviation
Choline chloride	Glucose	2:1	ChCl-Glu
	Glycerol	1:1	ChCl-G
	Ethylene glycol	1:2	ChCl-EG
	Urea	1:1	ChCl-U

Table 2.

Test set	Extraction conditions			Yield (mg/g sample)
	solvent to solid (g/g)	ultrasound power (w)	extraction time (min)	
1	-1(15)	-1(300)	0(50)	13.54±0.25
2	1(30)	-1(300)	0(50)	16.65±0.13
3	-1(15)	1(500)	0(50)	20.54±0.11
4	1(30)	1(500)	0(50)	18.24±0.16
5	-1(15)	0(400)	-1(40)	13.41±0.21
6	1(30)	0(400)	-1(40)	19.54±0.25
7	-1(15)	0(400)	1(60)	19.6±0.09
8	1(30)	0(400)	1(60)	14.36±0.11
9	0(22.5)	-1(300)	-1(40)	13.7±0.08
10	0(22.5)	1(500)	-1(40)	17.56±0.12
11	0(22.5)	-1(300)	1(60)	13.26±0.14
12	0(22.5)	1(500)	1(60)	16.15±0.10
13	0(22.5)	0(400)	0(50)	20.56±0.11
14	0(22.5)	0(400)	0(50)	21.06±0.18
15	0(22.5)	0(400)	0(50)	20.86±0.09
16	0(22.5)	0(400)	0(50)	18.51±0.17
17	0(22.5)	0(400)	0(50)	20.24±0.14

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818   Table 3.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	131.85	9	14.65	18.32	0.0005	significant
A- solvent to solid	0.36	1	0.36	0.45	0.5231	
B-ultrasound power	29.41	1	29.41	36.78	0.0005	
C-extraction time	0.09	1	0.09	0.11	0.7495	
AB	7.32	1	7.32	9.15	0.0193	
AC	32.32	1	32.32	40.41	0.0004	
BC	0.24	1	0.24	0.29	0.6044	
A^2	2.19	1	2.19	2.74	0.1417	
B^2	21.92	1	21.92	27.41	0.0012	
C^2	32.93	1	32.93	41.18	0.0004	
Residual	5.60	7	0.80			
Lack of Fit	1.45	3	0.48	0.46	0.7226	not significant
Pure Error	4.15	4	1.04			
Cor Total	137.45	16				

819   R^2=0.9593; R^2Adj =0.9069; R^2Pred =0.7844; Adeq Precision=10.479.

820   Table 4.

Extraction technology	Solvent composition	Solvent to solid ratio (g/g)	Ultrasound power (W)	Extraction time(min)	Yield of extract (mg/g)	UA	
						Yield (mg/g)	Purity (%)
UAE-DES -ATPS	36% (w/w) ChCl-Glu and 25% (w/w) K_2HPO_4	15:1	470	54	52.1±1.14	22.10±0.44	42.41±0.84
UAE-ethanol	85% ethanol (V/V)	15:1	470	54	103.6±0.85	20.9±0.79	20.17±0.77

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