Enabling technologies for the extraction of grape-pomace anthocyanins using natural deep eutectic solvents in up-to-half-litre batches extraction of grape-pomace anthocyanins using NADES

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33 **Enabling technologies for the extraction of grape-pomace anthocyanins using natural**

34 **deep eutectic solvents in up-to-half-litre batches**

35 **Extraction of grape-pomace anthocyanins using NADES**

36
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48 **Abstract**

49 Bioactive compounds should be extracted using alternative solvents and enabling

50 technologies, in accordance with green extraction principles. The aim of this study is to

51 develop an eco-friendly extraction method for grape-pomace anthocyanins on a larger scale.

52 From a preliminary screening of 8 different natural deep eutectic solvents (NADES), a

53 combination of choline chloride:citric acid was selected because of its price, physicochemical

54 properties, and anthocyanin recovery and stability. The effects of multimode-microwave

55 (MW), and low-frequency-ultrasound (US) irradiation (used alone or simultaneously), as well

56 as that of process parameters on extraction efficiency have been investigated in order to

57 maximise anthocyanin extraction yield. The best conditions were found to be: simultaneous

58 ultrasound/microwave-assisted extraction (UMAE) (MW power at 300 W, US power 50 W),

59 for 10 min with 30% (v/v) of water. This gave 1.77 mg g_{dw}^{-1} of anthocyanins. Anthocyanins

60 were efficiently recovered from NADES, which were recycled. The optimised procedure was

61 scaled up to a half-litre batch.

62

65
66 **1.   Introduction**

67 The development of new products, processes and sustainable technologies that minimise the

68 use of hazard chemicals is of growing interest to both industry and academia (Chemat et al.,

69 2012; Clarke et al., 2018). The huge impact of solvents in almost all industrial processes has

70 prompted the development of greener alternatives (Cvjetko Bubalo et al., 2018, 2015a;

71 Zainal-Abidin et al., 2017). Ideal solvents should be non-volatile (reduced air pollution), non-

72 flammable (process safety), stable (easier recycling and reuse) and based on renewable

73 sources. Natural deep eutectic solvents (NADES) are able to fully address all these

74 requirements. NADES are a new generation of chemicals with potential uses in various

75 industrial fields and have been dramatically expanding in popularity over the past few years.

76 NADES are a mixture of two or three components that form intramolecular hydrogen bonds;

77 hydrogen bond acceptors, such as organic salts (quaternary ammonium or phosphonium salt),

78 hydrogen bond donors (HBD) (sugar, alcohol, amino acid, organic acid etc.), and sometimes

79 up to 50 % (v/v) of water. Their numerous structural variations mean that it is possible to

80 tailor the physicochemical properties of NADES (Choi et al., 2011). They have therefore

81 attracted attention for their role as potential green solvents in many fields, including the

82 extraction of biologically active compounds from plant materials. Recent literature has

83 provided a number of examples of the NADES-mediated extraction of biologically active

84 compounds, especially polyphenols (Bosiljkov et al., 2017; Cvjetko Bubalo et al., 2018;

85 Jeong et al., 2015a; Zainal-Abidin et al., 2018). Polyphenols have successfully been extracted

86   from industrial by-products, such as wine lees, red grape pomace, onions, olives, tomatoes,

87   pear, and lemon-waste peels (Mouratoglou et al., 2016; Bosiljkov et al., 2017; Fernández et

88   al., 2018). The chemical properties of NADES make them well suited to MW irradiation

89   (Tommasi et al., 2017). However, all published research data to date refer to the lab scale,

90   and, to the best of our knowledge, there are no commercial NADES-based process in

91   existence. NADES show great potential for industrial application thanks to their acceptable

92   costs, the versatility of their physicochemical properties, simple and inexpensive preparation

93   and low cytotoxicity. However, the evident drawbacks caused by high viscosity and very low

94   vapour pressure (Bosiljkov et al., 2017) must be considered. Only Abbott et al. (2007) have

95   reported a scaled-up process that was performed using NADES choline chloride:glycerol

96   eutectic for biodiesel purification.

97   In this work, we aim to investigate the scale-up of NADES-assisted extraction and the

98   efficient final separation of target compounds from the extraction media. The experimental

99   design entails the following steps: (1) NADES selection; (2) technology selection and

100 extraction-parameter optimisation; and (3) the recovery of the target compounds and NADES

101 recycling (Fig. 1). Several NADES have been prepared and fully characterised. The most

102 promising NADES was selected for further optimisation according to physicochemical

103 characteristics, solvent price, anthocyanin extraction power and extract stability. The

104 influence of MW and US, used alone or simultaneously, was also investigated. The

105 parameters that influence the extraction process, such as time, water addition and power of

106 alternative energy sources, MW and/or ultrasound, were also studied and optimised using the

107 response surface methodology. Finally, the optimised method for the extraction of

108 anthocyanins from grape pomace was performed on a larger scale (0.5 L), with NADES

109 recycling. The experimental design, operational conditions and results are shown on Fig. 1. It

110 is expected that the extraction of anthocyanins with NADES, recovery of anthocyanins

111 and recyclaction of NADES are scalable.

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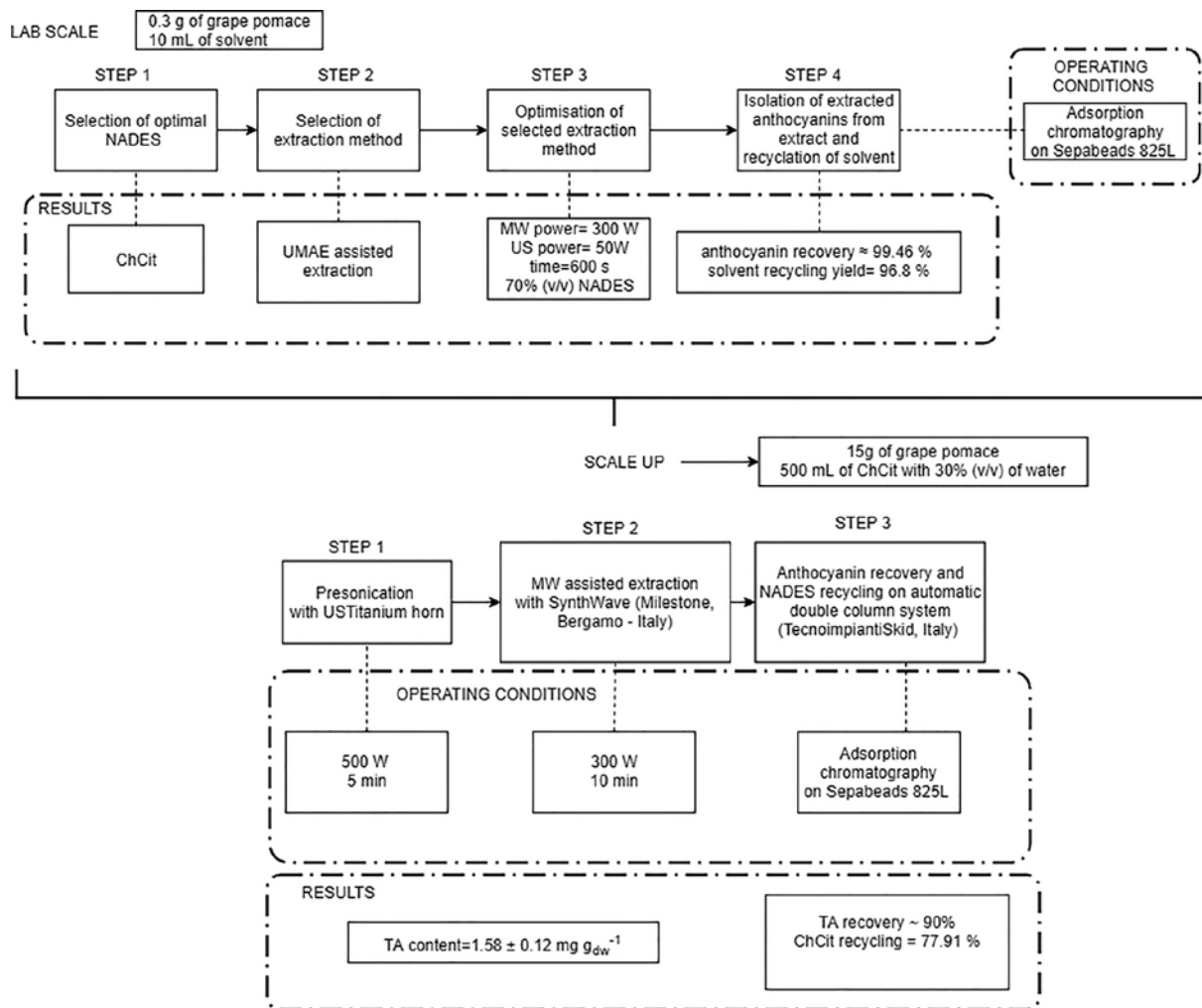
Fig. 1. Flowchart of experimental design, operational conditions and results

2. Materials and Methods

2.1. General

Citric acid, proline, betaine, malic acid, choline chloride, glycerol and glucose were purchased from Sigma (St. Louis, MO, USA). The standards (peonidin-3-*O*-monoglucoside, petunidin-3-*O*-monoglucoside, delphinidin-3-*O*-monoglucoside, malvidin-3-*O*-monoglucoside) were purchased from Polypolyphenols AS (Sandnes, Norway). Macroporous resin was provided by Tecno Impianti Srl, (Pozzuolo M.na – Milan, Italy).

Grape pomace was obtained from the Croatian native red grape cultivar, *Vitis vinifera* cv. Plavac mali, which originates from Dalmatia (Croatia southern vine- growing region). It was freeze-dried (Alpha 1-2 LD plus Christ, Germany) for two days, milled and stored at 25°C in a desiccator until extract preparation.

125   Software Statistica V.12 (Statsoft Inc., Tulsa, OK, USA) was used for the statistical analysis

126   of all experimental results.

127

128   ***2.2.Preparation and characterisation of NADES***

129   Choline chloride (ChCl) was dried in a vacuum concentrator (Savant SPD131DDA SpeedVac

130   Concentrator, Thermo scientific, USA) at 60°C for 24 h before use. The NADES were

131   synthesised at certain molar ratios of ChCl to hydrogen bond donor (HBD). The two or more

132   components were placed in specific ratios, with 10, 25, 30 or 50 % (v/v) of water, in a round-

133   bottomed glass flask, and then were stirred and heated to 50°C for 2 hours until a

134   homogeneous transparent colourless liquid was formed. NADES abbreviations and

135   corresponding mole ratios are given in Table 1.

136   The pH values for each NADES were determined using a 405-DPAS pH-electrode (Mettler

137   Toledo, Zagreb). The pH measuring range was 0–12 in a temperature range of 0–100°C.

138   Viscosities of prepared NADES were measured as described in Mitar et al., 2019.

139   The polarity of each NADES was determined using Nile red as a solvatochromic probe, as

140   described in Jeong et al. (2017) and Ogihara et al. (2004). Briefly, a Nile red solution (1.0 g L^{-}

141   ^{1}) was prepared and diluted 100 times in 96% (v/v) ethanol, and then added to the NADES,

142   after which the absorption spectra of the dye (λ_{max}) were measured using a GENESYS 10S

143   UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). The molar transition energy

144   (E_{NR}) was calculated using the following formula (Reichardt, 1994):

145    $$E_{NR}(kcal\ mol^{-1}) = \frac{h * c * N_A}{\lambda_{max}}$$

146   Where λ_{max} is maximum absorption wavelength, h is Planck's constant, c is the velocity of

147   light and N_A is Avogadro's constant.

148

149

Table 1. Used NADES (25 %, v/v) and their physicochemical properties.

NADES	Abbreviation	Molar ratio	Price (€kg⁻¹)[a]	pH	Polarity [E_{NR} kcal mol⁻¹]	Viscosity [Pas]
Choline chloride: Citric acid	ChCit	2:1	46.79	0.93	49.39	Anamarija
Choline chloride: Malic acid	ChMa	1:1	52.72	0.67	49.93	0.0179
Choline chloride: Proline: Malic acid	ChProMa	1:1:1	252.42	3.21	50.50	0.0121
Proline: Malic acid	ProMa	1:1	351.18	2.67	50.19	0.0150
Betaine: Malic acid	BMa	1:1	81.23	3.27	50.50	0.0462
Betaine: Citric acid	BCit	1:1	63.79	2.60	50.01	0.0554
Malic acid:Glucose:Glycerol	MaGlcGly	1:1:1	15.02	1.15	50.63	0.0447
Malic acid:Glucose	MaGlc	1:1	17.90	0.49	49.67	Anamarija

151   [a] The prices of solvents were estimated with reference to the website of Merck (Germany)
152
153   ***2.3. Selection of optimal NADES and extraction method***

154   Extraction was performed in a US bath XUB5 (XUB Series Digital Ultrasonic Baths, BioSan,

155   Latvia), equipped with Digital LCD controls, a timer and a heater (Heater power 150 W)

156   (Cvjetko Bubalo et al., 2016). All extractions were carried out under US (power 100 W) at

157   constant temperature (65°C) for 50 min. Solid-liquid ratios of 0.03 g of freeze-dried grape

158   pomace per mL of prepared NADES, which contained 25% (v/v) of water (Table 1) or

159   acidified aqueous ethanol (70% of ethanol with 0.1% of HCl, v/v), were used for extraction.

160   Extracts were then centrifuged for 15 min at 5000 g, the supernatant was decanted, adjusted to

161   a final volume of 10 mL (0.03 mg mL⁻¹) and stored at +4°C until further HPLC analyses were

162   performed (paragraph 2.8.).

163   The influence of US and MW, used alone and simultaneously, on extraction efficiency was

164   investigated. Experiments were performed in a MW-US-ER-01 cooperative extractor/ reactor

165   (LAB-KITS, China) for 10 min with US (40 kHz, 50 W) and/or MW (100 W) irradiation. 0.3

166   g of grape pomace was extracted with 10 mL of ChCit (25%, v/v). Extracts were then

167   centrifuged and the supernatant was stored and analysed as described above.

168   *2.4.Testing the stability of prepared extracts*

169   The storage stability of the prepared extracts was measured according to the procedure

170   reported by Dai et al. (2014).Briefly, extracts were stored in the dark at 25°C, 4°C and -18°C

171   for 60 days, and were monitored and analysed over the 60 days using HPLC (paragraph 2.8.).

172   Data are expressed as degradation rate (C/C_0), where C_0 is the initial anthocyanin

173   concentration and C is the anthocyanin concentration after incubation.

174

175   *2.5.Extraction method optimisation*

176   The optimisation of the UMAE extraction of grape-pomace anthocyanins was performed via

177   Box-Behnken design. The influence of the independent variables, MW power (X_1, 100–300

178   W), water content in NADES (X_2, 10–50%, v/v) and extraction time (X_3, 3–15 min), on the

179   dependant variable, extracted anthocyanin content (Y), was investigated (Table 1S). US

180   power was constant (50W) in all preformed experiments due to instrument set up, and the

181   maximum temperature was 80°C. Fifteen experiments were performed with 3 centre points

182   per block to optimise the extraction method, and the responses were fitted with a second-order

183   polynomial equation (RSM model) (Fernández et al., 2018).

184
$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad i < j$$

185   where $X_1, X_2,\ldots, X_k$ are the independent variables, Y the dependant variable, β_0, β_j (i = 1, 2,.. .

186   ., k), β_{ii} (i = 1, 2,. . ., k), and β_{ij} (i = 1, 2,. . ., k; j = 1, 2,. . ., k) are the regression coefficients

187   for the intercept, linear, quadratic and interaction terms, respectively, and k is the number of

188   variables.

189   Design-Expert (Version 7.0.0., Suite 480, Minneapolis, MN 55413) software was used for the

190   analysis of variance (ANOVA) to obtain the quadratic polynomial mathematical model, which

191   was established to describe the interaction of process parameters on extraction of total

192 anthocyanins .The value of the determination (R^2) and the model p value were used to predict

193 model capability.

194

195 **Table 1S.** Independent variables for the enperimental design

Independent variable	Variable levels			
	Symbol	Low (-1)	Center (0)	High (+1)
Power of microwave (W)	X_1	100	200	300
Water content in NAES (%, v/v)	X_2	10	30	50
Time (s)	X_3	600	750	900

196
197

198 *2.6.Experimental setup for extraction scale-up*

199 15 g of grape pomace were extracted using 500 mL of ChCit with 30% (v/v) of water in two

200 separate extraction steps. Initially, pomace extraction was performed in 5 minutes with a self-

201 made US Titanium horn prototype equipped with an US generator, and a US converter (20

202 kHz, power 500 W). Subsequently, grape pomace was extracted in the MW pressurised

203 reactor SynthWave (Milestone, Bergamo - Italy). The extraction was performed under

204 nitrogen pressure (3 bar) to avoid anthocyanin oxidation. The stirrer was set to 100%. A ramp

205 of 1 minute was set to increase the temperature up to 80°C, and extraction was then set for 9

206 more min at a power output of 300 W. The recovered extracts were centrifuged and the

207 supernatant was analysed by HPLC (paragraph 2.8.).

208

209 *2.7. Anthocyanin recovery and NADES recycling*

210 Anthocyanin recovery from the ChCit extracts was achieved via resin adsorption on a glass

211 column (1.1 cm x 11 cm), which was wet-packed with 6 g macroporous resin Sepabeads

212 SP207 (BV= 10.45 mL). Resin was pre-treated with 2 BV of 96% (v/v) ethanol and again

213 with 5 BV of deionised water according to Chen et al. (2015). The extracts prepared with

214 ChCit30, under optimal conditions on the lab scale, were diluted to 80% (v/v) of water and

215 flowed through the column at flow rate of 1.5 mL min^{-1}. ChCit was eluted from the column

216 with deionised water (3 BV) and the anthocyanins were desorbed using acidified aqueous

217 ethanol (75% (v/v) with 0.1% HCl (v/v)) (3BV). The water fraction was evaporated under

218 vacuum and ChCit was recovered. The cleanness of the NADES was checked by NMR

219 spectroscopy. ^{1}H NMR spectra from DMSO-d$_6$ were recorded on a Bruker AV-300

220 spectrometer, as described in Cvjetko Bubalo et al. (2015b).

221 EtOH fractions were analysed by HPLC, as described in paragraph 2.8, and yields were

222 calculated.

223 Experiments for the recovery of anthocyanins and the recycling of NADES on a larger

224 scale were performed on an automatic double column system ('green technologies

225 development platform', DSTF, University of Turin, Turin, Italy) by Tecnoimpianti Skid,

226 Italy (Cravotto et al., 2018) in one column (5 cm x 80 cm), equipped with 370 g of

227 Sepabeads SP825L (BV=884 mL), as described above with some changes. The flow rate was

228 15 mL min^{-1}.

229 Recycled NADES were diluted with 30% of water and used again for extraction.

230

231 *2.8. HPLC analyses*

232 HPLC analyses were performed as described in Panić et al. (2019). Briefly, extracts were

233 filtered through 0.22 µm PTFE (polytetrafluoroethylene) filters prior to the injection. The

234 concentration of anthocyanins was measured by Agilent 1200 Series HPLC system (Agilent,

235 San Jose, CA, USA) equipped with a diode array detector (DAD). The separation was

236 performed with Phenomenex C18 column (Kinetex 150 mm × 4.6 mm, 2.6 µm, 100 Å) at

237 40°C.

238 The injection volume was 10 µL. The mobile phases consisted of water/formic acid (95:5,

239 v/v) (solvent A) and acetonitrile/formic acid (95:5, v/v) (solvent B) with the following elution

240 gradient: 0-15.5 min, 10-35%; 15.5-16 min, 35%-100% B, 16-17 min, 100% B, 17-18 min,

241 100%-10% B, 18-22 min 10% B.

242 Anthocyanins were monitored at the wavelength of 520 nm at flow rate 0.9 mg mL^{-1} and

243 identification was based on the comparison of their retention times and UV-Vis spectral data

244 with external standards. The quantification was done with calibration curves of malvidin-3-*O*-

245 monoglucoside (3-300 mg L^{-1}).

246 Anthocyanins content was expressed as mg of malvidin-3-*O*-monoglucoside equivalent per g

247 of dry weight (dw). Total anthocyanins were expressed as a sum of identified anthocyanins.

248 HPLC analyses were conducted in triplicate.

249 2.9.Statistical analysis

250 The statistical analysis was performed using the software StatSoft, Inc. (2014). STATISTICA

251 (data analysis software system), version 12. The results were in the text and tables were

252 expressed as mean ± standard deviation (±SD). Differences between means were tested using

253 analysis of variance (ANOVA) at the significance level of $p < 0.05$ followed by the post hoc

254 Tukey's test. Response surface plots were drawn as a function of two factors while third

255 factor was constant with the.

256 **3. Results and discussion**

257 *3.1. NADES selection*

258 The first step in NADES selection is choosing the hydrogen bond donor and acceptor based

259 on the properties of the target compounds, namely grape-pomace anthocyanins. Anthocyanins

260 are highly polar compounds that are better solubilised in water than in non-polar solvents,

261 whose chemical form and stability depend on pH value. Anthocyanins are prevalent in the

262   flavylium cation form, which is stable at pH<2, while they change structure at increasing pH

263   values, and are degraded at pH >7 (Cvjetko Bubalo et al., 2016; Bosiljkov et al., 2017;

264   Cheynier et al., 2012). The literature indicates that organic acid-based NADES can fully

265   address the requirements of anthocyanin isolation and 8 different organic-acid based NADES

266   were prepared and characterised (Table 1) (Bosiljkov et al., 2017; Cvjetko Bubalo et al., 2018,

267   2016; Jeong et al., 2015b; Radošević et al., 2016).

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a

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b
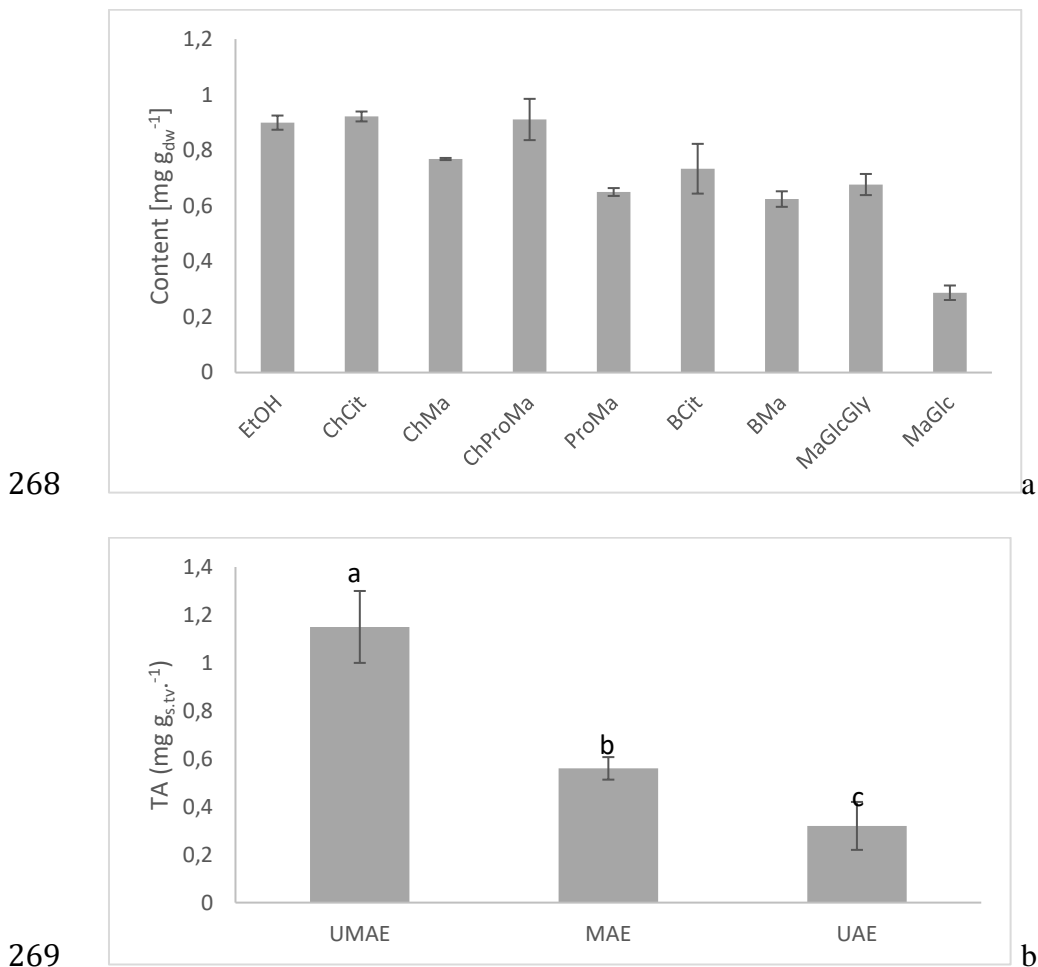
270   **Figure 2.** Total anthocyanins content (mg g^{-1} $_{dw}$) of prepared extract with different NADES

271   (a) and extraction efficiency for grape pomace anthocyanins with ChCit under different

272   irradiation (b). Total anthocyanins were expressed as a sum of identified anthocyanins.

273   Content of anthocyanins were expressed as the means (n=3) ± S.D. Presented value followed

274   by different lower-case letters (a-c) are significantly different ($p < 0.05$) as measured by

275   Tukey's HSD test.

276   All of the prepared solvents were polar, with polarities similar to water, and pH values were

277   from 0.49-3.27. NADES were prepared with 25% (v/v) of water in order to decrease viscosity

278   and improve mass transfer between the solid and liquid phases (Cvjetko Bubalo et al., 2018).

279   Results were also compared with acidified aqueous ethanol as the most suitable and green

280   conventional solvent (Makris, 2018) (Fig. 2.a). The highest content of total anthocyanins

281   (sum of identified anthocyanins by HPLC) was extracted with ChCit≈ChProMa≈EtOH,

282   followed by ChMa≈BCit≈MaGlcGly≈ProMa≈BMa>MaGlc. Furthermore, values ranged from

283   0.28   to

284   0.92   mg   g^{-1} dw,   indicating   great   variation   in   NADES   extraction

285   efficiency.   Although polarity and pH values were previously highlighted as the most

286   important solvent characteristics for anthocyanin extraction, no obvious correlation was

287   observed (Radošević et al., 2016). It seems that viscosity is the major property that influences

288   extraction efficiency for organic acid based NADES since an obvious relationship was found;

289   extraction efficiency increased as solvent viscosity decreased. Furthermore, the obtained

290   anthocyanin profiles in extracts were in good agreement with literature; four anthocyanin-3-

291   O-monoglucosides (delphinidin, petunidin, peonidin, malvidin), two acylated derivatives

292   (malvidin- and peonidin-3-acetylmonoglucosides) and two coumaroyl derivatives (peonidin-

293   and malvidin-3-(6-O-p-coumaroyl) monoglucosides) were observed, with malvidin-3-O-

294   monoglucoside being most abundant (Kammerer et al., 2004; Teixiera et al., 2014). A

295   comparison of conventional solvent and NADES highlighted that the latter showed higher

296   extraction efficiency for anthocyanin-3-O-monoglucosides, while anthocyanin-3-(6-O-p-

297   coumaroyl) monoglucosides were displayed improved extraction with acidified ethanol. The

298   different selectivities of anthocyanin extraction with NADES and conventional solvents have

299   already been published (Dai et al., 2016) (Table 2S). As the extraction efficiency was not

300   significantly different (p<0.005) for ChCit, ChProMa and acidified aqueous ethanol, the

301   stability of anthocyanins in those solvents at -18°C, 4°C and 25°C for 60 days was also

302 considered prior to NADES selection. This is especially interesting because extracts that

303 were prepared with NADES can be considered safe for further application without expensive

304 downstream purification (Radošević et al., 2016) (Fig. 3) Generally, no differences in

305 anthocyanin stability in the various solvents were observed at room temperature, while ChCit

306 was found to possess the highest stabilising capacity at 4°C and -18°C. At 4°C, 70% of

307 anthocyanins were degraded in ChProMa, while in ChCit it was only 14%.

308

309 **Table 2S.** Anthocyanins content (mg g^{-1} dw of pomace) of prepared extract with different NADES. Content of anthocyanins were expressed as the

310 means (n=3) ± S.D.

Prepared extracts	delphinidin-3-*O*-monoglucoside	petunidin-3-*O*-monoglucoside	peonidin-3-*O*-monoglucoside	malvidin-3-*O*-monoglucoside	peonidin-3-*O*-acetylmonogluc oside	malvidin-3-*O*-acetylmonogluc oside	cyanidin -3-(6-*O*-p-coumaroyl)mon oglucosides	peonidin-3-(6-*O*-p-coumaroyl)mon oglucosides	malvidin-3-(6-*O*-p-coumaroyl)mon oglucosides
EtOH	22.1±3.30	32.2±4.18	21.5±2.69	226.7±10.14	37.9±0.61	33.1±1.73	53.5±4.96	40.6±1.02	444.9±2.30
ChCit	32.8±0.34	48.5±0.01	22.8±0.43	285.6±5.45	37.5±0.54	34.3±1.67	45.5±0.93	36.8±0.97	387.0±7.52
ChMa	26.2±0.81	40.3±1.27	18.2±0.58	239.1±2.66	30.0±0.25	29.6±0.80	36.0±1.06	29.4±0.07	317.5±6.94
ChProMa	50.03±0.00	49.2±31.82	25.5±2.48	285.0±24.75	28.8±2.48	27.8±2.48	44.8±2.48	34.6±2.48	403.3±2.48
ProMa	31.02±0.00	35.9±1.77	9.6±1.77	221.5±1.77	6.8±1.77	23.9±1.77	26.2±1.77	17.8±1.77	285.3±1.77
BCit	21.6±1.23	32.3±16.54	15.7±9.00	180.9±93.16	24.5±12.55	20.7±10.63	29.0±14.21	23.6±12.05	235.8±109.13
BMa	27.1±0.06	38.9±1.54	20.8±1.21	190.2±1.99	30.8±0.66	26.3±0.30	35.7±2.09	29.4±0.40	239.5±22.15
MaGlcGly	25.5±1.18	37.8±1.99	17.1±0.17	214.0±8.51	26.5±1.50	22.9±0.64	31.5±2.14	24.9±1.39	256.8±20.52
MaGlc	10.4±1.16	15.5±1.71	6.7±0.70	88.2±8.93	11.0+1.00	9.3±0.89	13.1±1.21	10.2±1.00	109.3±9.52

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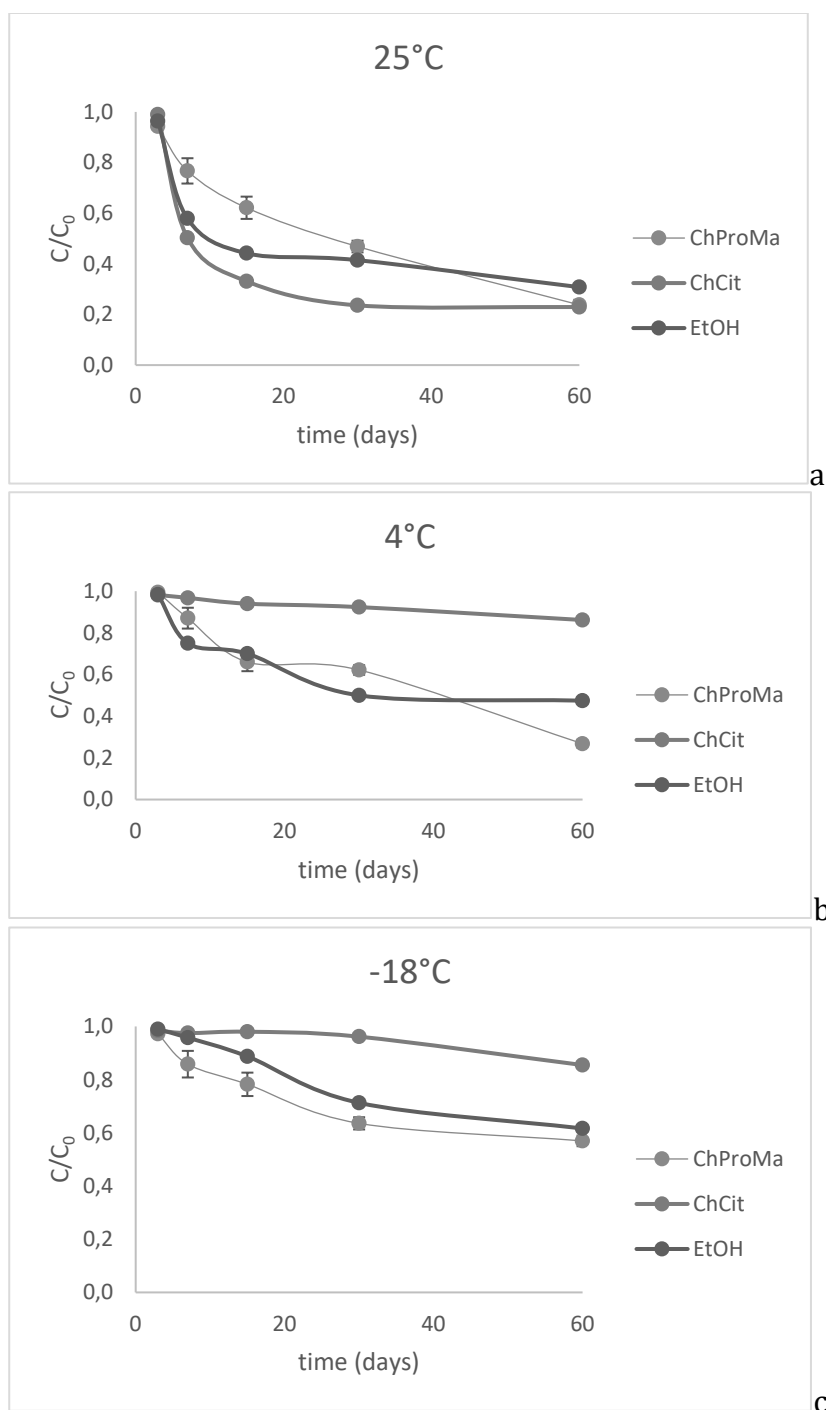
Figure 3. Degradation curve of anthocyanins in ChProMa- ChCit- and EtOH- extracts at 25°C (a), 4°C (b) and -18°C (c) through 60 days. Results were expressed as the means (n=3) ± S.D

Similarly, it was noticed that only 10% of anthocyanins were degraded at -18°C in the ChCit-extract. Dai et al. (2016) have also noticed the higher stability of polyphenols in NADES than in conventional solvents.

Solvent cost is an important factor in scale-up development. The price of NADES is generally similar to that of organic solvents (~50 €kg^{-1}), but NADES structure can vary considerably. The cost of our tested mixtures ranged from 15.02 €kg^{-1} (MaGlcGly) to 351.18 €kg^{-1} (ChProMa). An analysis of all observed criteria (physicochemical characteristics, extraction efficiency, stability of anthocyanins and solvent cost) led to ChCit being selected as the solvent for the isolation of grape-pomace anthocyanins, and it was thus used in further experiments.

3.2. From small-scale experiments to the half-litre batch

After NADES selection, the eco-friendly extraction method was optimised and scaled up. Innovative technologies (US, MW, and UMAE) were considered as a means to reduce energy consumption and increase extraction efficiency. Non-conventional enabling technologies have found several potential applications in food industries thanks to their higher efficiency and energy savings (Barba et al., 2016). Furthermore, NADES are considered to be potential green alternatives to organic solvents in the extraction of bioactive molecules, and are compatible with those technologies. Grape-pomace extracts in ChCit with 25% (v/v) of water were prepared using a range of techniques. Anthocyanin contents were extracted in the following order, highest to lowest, UMAE>MAE>UAE (Fig. 2.b). UMAE irradiation was therefore selected as the extraction method. Process parameters were subsequently optimised using response-surface-methodology based experimental design. Software package experiments were designed using Design Expert 7.0.0., results were evaluated and the extraction method was optimised. Extraction time, power of MW irradiation and water content in the solvent were observed to have the highest impact on efficiency, and those conditions were optimised (MW power was tested from 100 – 300 W, water content in NADES from 30-50% v/v, and extraction duration from 600-900 s). MW power and extraction time were optimised in order to reduce energy consumption and prevent anthocyanin degradation. The

water content in NADES was optimised in order to manage viscosity, but retain extraction efficiency.

Box-Behnken design was successfully used to evaluate the influence of UMAE process parameters; the response (content of total anthocyanins) and model design are shown in Table 2. The content of total anthocyanins ranged from 0.1 to 1.8 mg g^{-1} of dw. In order to obtain the significant effects of the independent variables on response, the designed experiments were performed with the central values for MW power, 200 W, an extraction time of 12.5 min and a water content in NADES of 30% (v/v) (Table 1S). Furthermore, the RSM model was evaluated and analysis of variance (ANOVA) was used to calculate the statistical significance of the quadratic model.

Table 2. Experimental matrix and values of observed response.

Number of experiment	Variable X_1	Variable X_2	Variable X_3	Response
	P [W]	t [s]	%NADES [%, w/w]	w [TA] [mg g$_{dw}^{-1}$]
1	100	600	70	0.96
2	100	900	70	1.41
3	300	600	70	1.8
4	300	900	70	1.53
5	200	600	50	1.14
6	200	900	50	0.95
7	200	600	90	0.1
8	200	900	90	0.63
9	100	750	50	0.67
10	300	750	50	1.14
11	100	750	90	0.05
12	300	750	90	0.5
13	200	750	70	1.15
14	200	750	70	1.09
15	200	750	70	1.19

* MW power – P, time – t, content of ChCit - %NADES, w [TA]- total anthocyanin content

According to the results of the multiple regression analysis presented in Table 3S, the relationship between the independent variables and the response can be expressed, in coded values, as:

$$Y_1 = 1{,}14 + 0{,}09 * X_1 + 0{,}26 * X_2 - 0{,}33 * X_3 - 0{,}23 * X_1 * X_2 + 0{,}18 * X_1 * X_3 - 0{,}005 * X_2 * X_3 + 0{,}18 * X_1{}^2 + 0{,}058 * X_2{}^2 - 0{,}61 * X_3{}^2$$

where, Y_1 is the content of grape-pomace anthocyanins, X_1 is MW power, X_2 is time of extraction and X_3 is the water content in NADES.

Table 3S. Analysis of variance (ANOVA) of the modelled responses.

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value
Model	3.39	9	0.38	135.46	<0.0001
X_1	0.064	1	0.064	23.05	0.0049
X_2	0.54	1	0.54	195.19	<0.0001
X_3	0.85	1	0.85	306.56	<0.0001
$X_1 X_2$	0.21	1	0.21	76.76	0.0003
$X_1 X_3$	0.13	1	0.13	46.68	0.001
$X_2 X_3$	0.0001	1	0.0001	0.036	0.8571
$X_1{}^2$	0.11	1	0.11	39.67	0.0015
$X_2{}^2$	0.012	1	0.012	4.48	0.088
$X_3{}^2$	1.38	1	1.38	495.55	<0.0001
Residual	0.014	5	0.00278		
Lack of fit	0.00886	3	0.00295	1.17	0.4919
Pure error	0.00505	2	0.00253		
Total	3.41	14			
R^2	0.9960				

*P < 0.01 highly significant; 0.01 ≤ P < 0.05 significant; P ≥ 0.05 not significan*
X1: microwave power; X2: time; X3: ChCit concentration

The determination coefficient (R^2) of 0.9960 indicates that the quadratic regression model was a good fit for the extraction of grape-pomace anthocyanins with NADES in UMAE conditions. The F-value (135.46), with a very low *p*-value (<0.0001), of the model indicated that the quadratic model was statistically significant. According to the *p*-values, which were less than 5% for all

variables, except $X_2 X_3$ and X_2^2, the variables had a statistically significant influence on Y_1; the content of grape-pomace anthocyanins (Table 3S).

Furthermore, 3D response-surface plots were created as function of two independent variables on fixed values (one independent and response) (Fig. 4). The significant influence that MW power had on total anthocyanin content is demonstrated in Fig. 4 (a, b). Total anthocyanin content increases with increasing MW power. Water content also had a significant effect on the extraction efficiency of total anthocyanins. The response-surface plots showed that total anthocyanin content increased with increasing water content, from 10 to 30%, while further increases led to decreases in total anthocyanin content, as has been previously noted (Bosiljkov et al., 2017).

It seems that mass transfer between the solid and liquid phases is hampered by high solvent viscosity in NADES with 10% water, while anthocyanins are much less stable in NADES with higher water contents. Dai et al. (2014) have also reported on the relationship between water content in NADES and anthocyanin stability, while Dai et al. (2013) and Nam et al. (2015) noticed that solvent viscosity is influenced by water content, and thus impacts upon mass transfer. MW and US irradiation also decrease the viscosity of NADES. Extraction time had a significant effect on total anthocyanin content. Increasing the extraction time led to decreased anthocyanin content, indicating that long extraction times and prolonged heating increase degradation (Cravotto et al., 2018). The optimal values for the maximum response obtained using the Box-Behnken method was 1.8 mg g^{-1}_{dw}. The independent variables were: X_1= 300 W, X_2=600 s and X_3=30% of water in NADES (%). The verification and validity of the generated mathematical model were performed via the extraction of anthocyanins at these optimal conditions. After performing that experiment under the optimal conditions, extraction efficiency was 1.77 mg g^{-1}_{dw}.
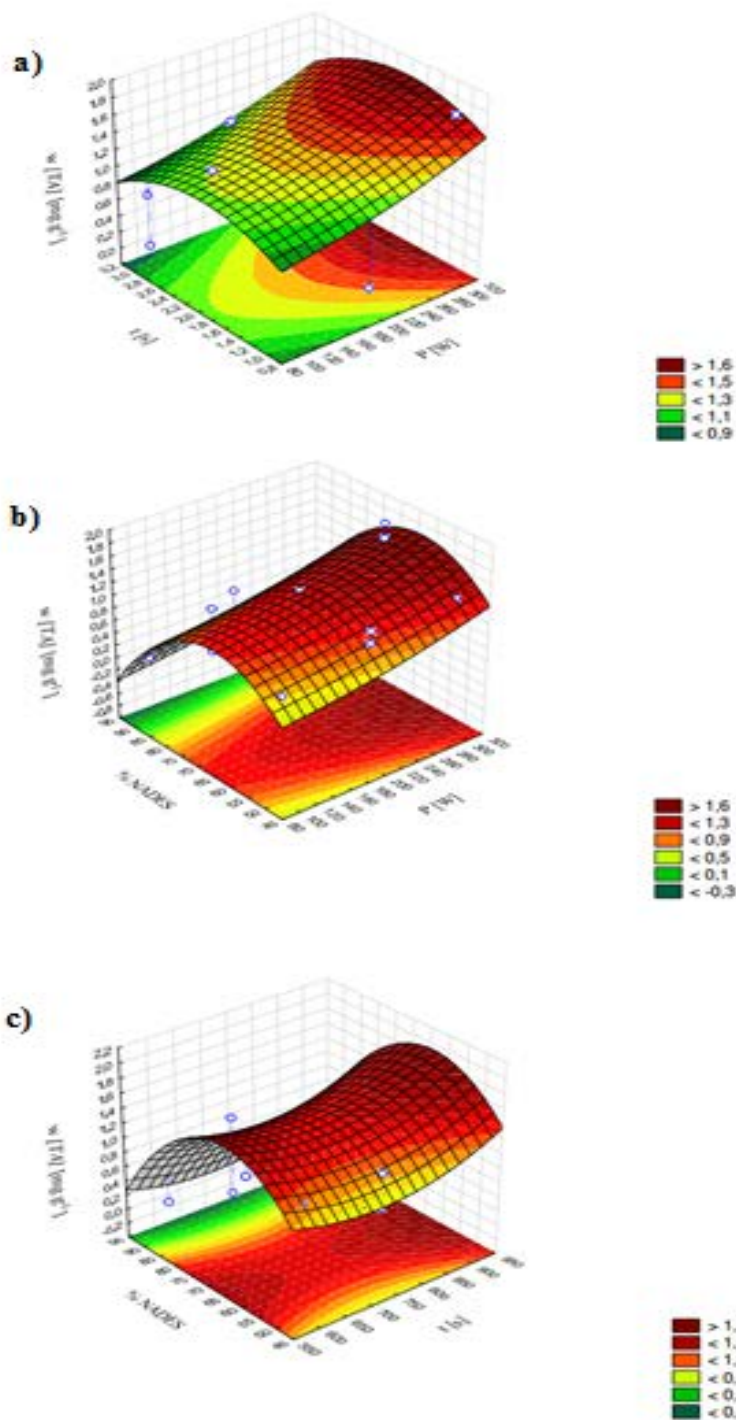
Figure 4. Response surface plots showing combined effects of microwave power and time (a), microwave power and NADES concentration (b) or NADES concentration and time (c) on content of total anthocyanins.

UMAE reactors present several setup possibilities (Chemat and Cravotto, 2012; Leonelli and Mason, 2010). Although UMAE extraction can be achieved by inserting a non-metallic horn or US transducer into a MW oven, that only works on the lab scale (Cravotto and Cintas, 2007). A second possible instrument setup for UMAE on a larger scale is an US–MW flow reactor with sonication outside the MW oven. Cravotto et al. (2007) have successfully exploited flow reactors for the treatment of polluted water. However, it appears that pumping solid particles through the system for solid-liquid extraction is impossible. Sequential US and MW irradiation in batch reactors may therefore be the most promising tool for industrial plant-extraction applications. Double irradiation can have synergistic effects on extraction processes and can double extraction efficiency; sonication can rupture cells, while MW promotes the release of the target compounds into the solvent (Chemat and Cravotto, 2012).

In this work, extraction was performed on a larger scale over 2 separate steps; US pretreatment, in the form of the sonication of grape pomace in ChCit (500 W, 5 min), before MW-assisted extraction (300 W, 10 min, 30% of water in NADES). Under such conditions, 1.58 ± 0.12 mg g_{dw}^{-1} of anthocyanins were extracted, which is equal to laboratory-scale results, indicating that the NADES process is promising for extraction on larger scales.

3.3. Recovery of anthocyanins and NADES recycling

Polyphenolic extracts that were prepared in NADES can be considered ready-to-use in the food and pharmaceutical industries, without the need for demanding and expensive downstream purification steps (Jeong et al., 2017; Radošević et al., 2016). However, the final goal is sometimes to have pure target compounds for further industrial applications. Furthermore, solvents should be recycled after processing. Organic solvents that are used for extraction are usually recycled via distillation. Volatile organic solvents are a major source of industrial waste (Sheldon, 2016). On the other hand, NADES have very low vapour pressure (Cvjetko Bubalo et al., 2018, 2015a), making them very difficult to evaporate for the easy

isolation of target compounds, which may be a problem for industrial applications. Current literature suggests several possibilities for target-compound recovery and NADES recycling. These include liquid−liquid extraction using another solvent, solid−liquid extraction using a macroporous resin, and the addition of antisolvents (Jeong, et al., 2015b; Ruesgas-Ramon et al., 2017). Adsorption chromatography was used in this work, with macroporous resin Sepabeads 825L as the stationary phase. From the grape-pomace extract that was prepared with ChCit under optimum conditions on the lab scale, as mentioned above, anthocyanins were adsorbed onto a resin, NADES was eluted with deionised water, and anthocyanins were desorbed with acidified, aqueous ethanol (after 3 BV). The recovery yield of anthocyanins was 70.36% and the solvent recycling yield was 94.78%, but NADES still retained some colour. Since our final goal was the complete recovery of anthocyanins, as well as the recycling and reuse of the solvent for further extraction, a recovery yield of ~70% was not deemed to be satisfactory, and a number of ways to improve recovery were considered. Our hypothesis was that recovery yields would be higher if the bonds between NADES and target compounds were broken. As reported in Cvjetko Bubalo et al., 2015a, the bonding of molecules into the structure of NADES has been observed. Therefore, the grape-pomace extract in ChCit was diluted to 80% of water prior to adsorption chromatography as the presence of > 50 % (v/v) of water ruptures the NADES structure (Yang, 2018). Under these conditions, anthocyanin recovery was highly efficient at ≈ 99.46 %, and was followed by highly efficient solvent recycling (yield 96.8 %). The cleanness of the NADES after recycling was checked using NMR spectrometry. The ^{1}H NMR spectra of freshly synthesised and recycled NADES were recorded for that purpose (Fig. 1S) and no significant differences in ^{1}H NMR spectra were observed. Only the chemical shift characteristics of NADES were found on ^{1}H NMR spectra, indicating the cleanness of recycled NADES. This implies that NADES structures should be disturbed prior to recycling via dilution with > 50% of water (v/v) in order to release anthocyanins and make them available for better adsorption on macroporous resins.
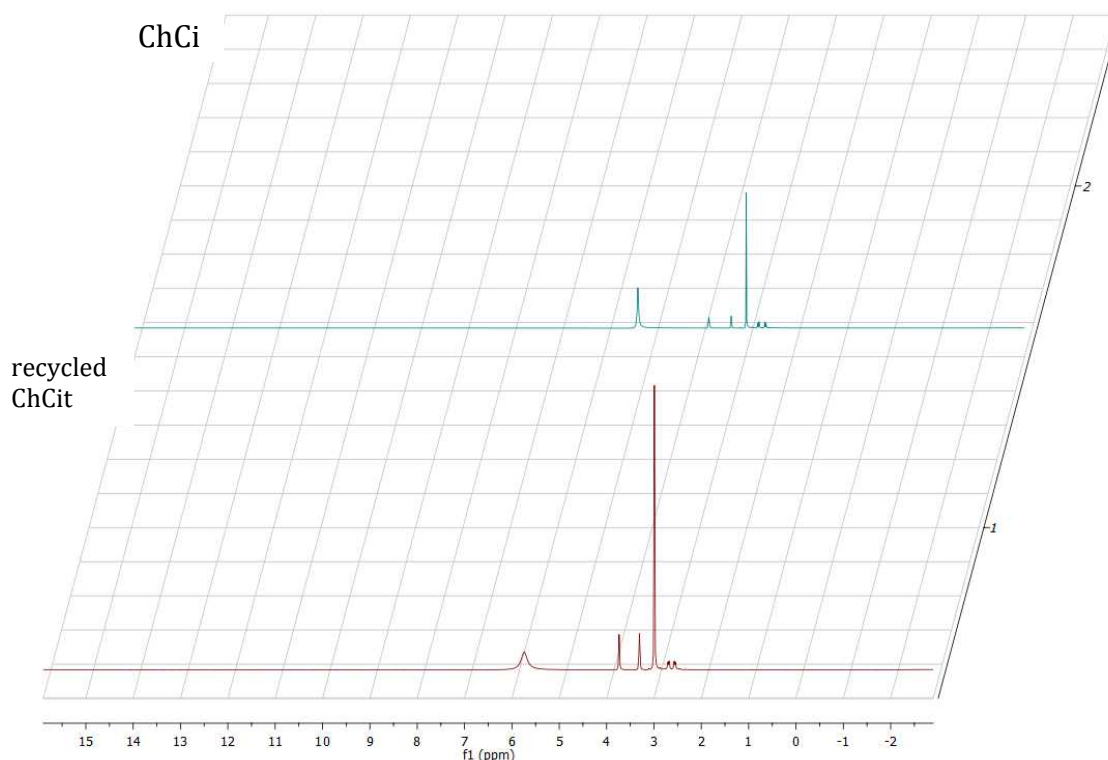
Figure 1S. ^{1}H NMR spectra of fresh and regenerated NADES ChCit.

Based on the results above, a method for anthocyanin recovery and NADES recycling was also performed on a pilot scale in an automatic double-column system that was presented in Cravotto et al., 2018. Anthocyanins from an extract that was prepared on a larger scale were recovered and the NADES was recycled. The recycling yield of ChCit in this system was 77.91 %, and anthocyanin recovery was ~ 90%. By comparison, Zhuang et al. (2017) and Wang et al. (2017) recovered 77.4-98.2% and 75.3-85.5% of polyphenols, respectively, on a macroporous resin from a plant extract that was prepared with NADES.

Furthermore, the regenerated solvent was then used for the extraction of grape-pomace anthocyanins. Extraction efficiency with the recycled solvent was 1.42 ± 0.11 mg g^{-1}, 11% less than that of freshly synthesised NADES. Similarly, an 8.1% lower amount of ginsenosides has been extracted using recycled NADES than with the original solvent system (Jeong, et al., 2015b). However, our results indicate that the NADES extraction method is scalable as is anthocyanin

recovery and solvent recycling. This study is a first step towards the implementation of this method on the industrial scale.

4. Acknowledgements

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