

# Extraction of Bioactive Compounds from *Red Cardinal* Grape Pomace by Deep Eutectic Solvents

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The objective of this study was to determine the most suitable extraction conditions from *Red cardinal* grape pomace using deep eutectic solvents (DES). Factors influencing the extraction process were investigated, including DES type, the ratio of hydrogen bond acceptor (HBA) to hydrogen bond donor (HBD), water addition, solvent-to-material ratio, extraction temperature, and time. The best results were obtained with choline chloride: glycerol: citric acid (1:1:1, w/w/w) DES, adding 25 % water, using a 25:1 (v/w) solvent-to-material ratio, 60 °C, and 2.5 h extraction. The grape pomace extract showed substantial levels of bioactive compounds, with the highest recorded values being  $65.49 \pm 1.30$  mg GAE/g DW for total polyphenol content,  $177.88 \pm 5.17$  mg AE/g DW for total triterpenoid saponin content, and  $36.22 \pm 0.77$  mg CE/g DW for proanthocyanidin content. The concentrations of specific compounds were quantified, revealing DES's superior extraction compared to ethanol and methanol, highlighting DES's effectiveness as an alternative extraction solvent.

## 1. Introduction

Grape cultivation is a significant global agricultural industry, producing approximately 74.8 million t annually in 2021. Grapes used for wine and juice production account for 51.2 % of the total grape production for processing (OIV, 2021). The main by-product of wine and juice production is the pomace, constituting about 15-20 % of the total weight of wine grapes. Studies have shown that grape pomaces (GP) retain significant amounts of bioactive compounds after winemaking (Bordiga et al., 2019). The recovery of bioactive compounds from GP presents an economically viable opportunity and supports the sustainable development of wine and juice production systems.

Bioactive compounds have been extracted using conventional organic solvents. These solvents have several drawbacks, including flammability, low biodegradability, toxicity, and volatility. In light of these concerns and the need for more environmentally-friendly approaches, there has been considerable attention towards alternative green solvents. Deep eutectic solvents (DES), considered Generally Recognized as Safe (GRAS) solvents, have emerged as potential green solvents in recent years. These solvents consist of a blend of hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD). DES possesses impressive properties, such as ease of preparation, low volatility, biodegradability, and non-toxicity (Omar and Sadeghi, 2023).

Numerous studies demonstrate that DES exhibit distinctive physicochemical properties, i.e. the capacity for viscosity adjustment (Romero-Rueda and Macias-Salinas, 2023), a broad polarization range, and remarkable solubility across a diverse array of compounds (Iannone et al., 2021). These attributes warrant comprehensive investigation, involving an exploration of the types of DES formulations stemming from natural HBA and HBD, their respective ratios, the volume of water introduced, and the specific extraction conditions encompassing parameters such as solvent-to-material ratio, temperature, and duration.

The principal aim of this research was to identify the variables affecting the extraction of bioactive compounds from GP using DES and to compare DES with traditional chemical solvents. By exploring these factors, the research aims to contribute to the advancement of sustainable and environmentally friendly extraction methods for the grape processing industry.

## 2. Materials and Methods

### 2.1 Material and chemicals

*Red cardinal* GP was sourced from Ba Moi Company, in Ninh Thuan province, Vietnam. The collected GP was separated into branches and seeds, dried using a heat-pump dryer at 40 °C until the moisture content reached 10 % or lower, ground into a fine powder ( $\leq 500 \mu\text{m}$ ), packed in PE/PA bags, and stored at -18 °C for subsequent experiments. The chemicals utilized were of analytical grade. The standards included: escin (Sigma-Aldrich, USA), catechin (Thermo Fisher Scientific, UK), kaempferol and rutin (The Institute of Drug Testing of Ho Chi Minh City, Vietnam), resveratrol and myricetin (Cool Chemistry, China), epicatechin (Sigma-Aldrich, USA), and gallic acid and quercetin (Himedia, India).

### 2.2 Preparation of DESs

Seven different DESs were employed: Choline chloride: Citric acid (ChCl-CA, 2:1, w/w), Choline chloride: Glycerol: Citric acid (ChCl-Gly-CA, 1:1:1, w/w/w), Choline chloride: Glycerol: Lactic acid (ChCl-Gly-LA, 1:1:1, w/w/w), Choline chloride: Glycerol: Malic acid (ChCl-Gly-MA, 1:1:1, w/w/w), Choline chloride: Glycerol (ChCl-Gly, 1:2, w/w), Choline chloride: Ethylene glycol (ChCl-EG, 1:2, w/w), and Choline chloride: 1,4 Butanediol (ChCl-1,4 But, 1:2, w/w). These DESs were formulated using the method following Ali et al. (2019) with slight adaptations: heated at 80 °C for 1.5 h on a magnetic stirrer until a clear solution was formed, followed by the addition of water for homogeneity.

### 2.3 Utilization of DES for extraction

For the extraction process, about 1 g of GP powder was mixed with each DES, using a solvent-to-material ratio of 15:1, 20:1, 25:1, and 30:1 (v/w). The mixture was stirred continuously on a magnetic stirrer at 260 rpm and maintained at different temperatures (30, 40, 50, 60, and 70 °C) for different extraction times (1.5, 2.0, 2.5, and 3 h). After extraction, the mixture underwent centrifugation at 6,000 rpm for a duration of 10 min, with the temperature rigorously kept at 4 °C. The resulting supernatant, which held the desired extracts, was separated from the sediment. The enriched supernatant was then used for analyzing total polyphenol content (TPC), total triterpenoid saponin content (TSC), and proanthocyanidin content (PAC). The best extraction conditions from the previous experiment were selected for the subsequent trial.

### 2.4 Comparison

For comparison, four distinct extraction conditions were conducted to recover bioactive compounds from the same batch of GP powder. Within this set, two extraction conditions involved the employment of DES under the most suitable extraction conditions within the scope of this study and a 50 % ethanol, a temperature of 50 °C, a solvent-to-material ratio of 15:1 (v/w), and an extraction time of 2 h. The remaining two extraction conditions applied to GP powder followed the methodologies outlined by Caldas et al. (2018) for ethanol-based extraction and Iglesias-Carres et al. (2018) for methanol-based extraction.

### 2.5 Analyses

#### 2.5.1 Total polyphenol content

Approximately 0.5 mL of each diluted extract was transferred to individual test tubes. To each tube, 0.5 mL of 10 % Folin-Ciocalteu solution was added and thoroughly mixed. After 5 min, 2.5 mL of 20 %  $\text{Na}_2\text{CO}_3$  was added, and the tubes were shaken again. The reaction tubes were then incubated at ambient temperature for 1.5 h to allow the chemical reactions to take place fully. The absorbance of the resultant solution in each test tube was measured at 765 nm using a spectrophotometer. TPC was quantified as mg of gallic acid equivalent per g of dry weight of GP powder (mg GAE/g DW) using gallic acid as the standard (Škulcova et al., 2017).

#### 2.5.2 Total saponin content

TSC was quantified following the method outlined by Tan et al. (2014). A mixture of 8 % (w/v) vanillin solution (0.3 mL) and 72 % (v/v) sulfuric acid (3 mL) were combined with the extract (0.3 mL). This resultant mixture was then kept at 60 °C for 15 min. The solution's absorbance was assessed at 560 nm. Aescin was employed as the standard to quantify the TSC, and the values were reported as mg of Aescin equivalent per g of dry weight of GP powder (mg AE/g DW).

#### 2.5.3 Total proanthocyanin content

The extract (0.5 mL) was mixed with 0.3 mL of 4 % (w/v) vanillin solution and 1.5 mL of 36 % HCl. The resultant mixture was incubated at ambient temperature for 15 min. Afterward, the absorbance of the solution was

assessed at 500 nm. Catechin was used as the standard to quantify the PAC, and the results were reported as mg catechin equivalent per dry weight of GP powder (mg CE/g DW) (Li et al., 2006).

### 2.5.4 Individual phenolic compounds

Individual phenolic constituents were analyzed using a HPLC system (Shimadzu SPD-20A HPLC system (Shimadzu, U.S.A.)) with an Inertsil ODS-3 Column (4.6 x 250 mm, 4  $\mu$ m, GL Sciences, Japan), and the amounts were expressed as mg/g DW. The mobile phases utilized in this process encompassed (A) a solution of 0.5 % formic acid and (B) 100 % acetonitrile. The elution program: 0-25 min, 10-30 % B; 25-40 min, 30-40 % B; 40-50 min, 40-50 % B; 50-55 min, 50-10 % B; 5 min equilibration. A volume of 10  $\mu$ L sample was injected, and the flow rate was kept 1.0 mL/min. Detection of specific phenolic compounds occurred at distinct wavelengths: catechin, epicatechin, gallic acid, kaempferol, myricetin, resveratrol, and rutin were detected at 280 nm, while quercetin was detected at 360 nm.

### 2.6 Statistical analysis

The experiments and consequent analyses were executed in triplicate, with the results reported as mean values accompanied by their respective standard deviations. The individual factor experiments were performed in a randomized manner, aiming to determine the impact of DES type, HBA/HBD ratio, water addition, a solvent-to-material ratio, temperature, and time on the extraction yield of TPC, TSC, and PAC. A separate one-factor experiment was carried out to compare the effects of different solvents. The values were subjected to analysis of variance and the least significant difference, accomplished through JMP 11.0 software, to identify significant disparities.

## 3. Results and Discussion

### 3.1 The effect of DESs type

The selection of DES (with 30 % water addition, solvent-to-material ratio of 20:1 (v/w), 30 °C, and 1.5 h extraction time) used as the extracting solvent significantly impacted the total bioactive compounds in GP (Figure 1). The cumulative "Total" value is the sum of the individual TPC, TSC, and PAC quantities. DES-extracted samples showed higher bioactive compound levels than other solvents. ChCl-Gly-CA exhibited the highest TPC, TSC, and PAC values:  $29.82 \pm 0.61$  mg GAE/g DW,  $66.33 \pm 0.81$  mg AE/g DW, and  $11.76 \pm 0.35$  mg CE/g DW. This DES outperformed others significantly. The extraction efficacy from plant materials depends on DES type. More polar solvents yield higher quantities of polar molecules, influencing extraction efficiency across the seven solvents. Solvent acidity is crucial in extraction selectivity (Dabetić et al., 2020), with bioactive compound extraction improving with increased solvent acidity. ChCl-Gly-CA superior extraction aligns with previous studies highlighting acid-based DESs' efficient phenolic compounds extraction due to higher polarity and functional group specifics. The OH functional group's prevalence in glycerol notably enhances hydrogen bonding within DES structure (Silva et al., 2020).

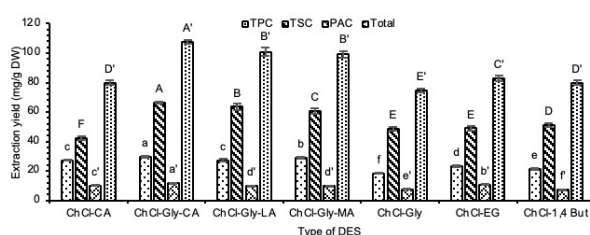


Figure 1: The influence of the specific DES type on the extraction yield of bioactive compounds

### 3.2 The effect of the HBA/HBD ratio and water addition

The optimal DES molar ratio selection critically impacts effective separation process. In ChCl-Gly-CA based DES, ChCl served as HBA, with Gly and CA as HBDs. TPC, TSC, and PAC showed significant variations with an increase in HBAs or HBDs in the DES. The TPC, TSC, and PAC were the highest at a 1:1:1 (w/w/w) ratio of ChCl-Gly-CA, yielding  $31.04 \pm 0.73$  mg GAE/g DW,  $66.51 \pm 1.07$  mg AE/g DW,  $12.57 \pm 0.33$  mg CE/g DW (Figure 2a). The increase in HBA/HBD ratios had a notable effect on the DES structure, affecting the extraction process. The distinct structure of DES's promotes efficient analyte extraction via intermolecular hydrogen bonding between the DES and solutes. Selecting an ideal molar ratio for extraction requires the consideration of various factors, including viscosity, hydrogen bonding, density, and electrical conductivity (Omar and Sadeghi, 2023), and the stability of the DES structure (Bildik, 2021).

At 25 % water addition, TPC, TSC, and PAC reached peak values:  $33.15 \pm 0.56$  mg GAE/g DW,  $69.44 \pm 0.89$  mg AE/g DW,  $11.76 \pm 0.14$  mg CE/g DW (Figure 2b). As the water addition was increased from 25 % to 40 %, TPC, TSC, and PAC showed significant differences, decreasing from  $114.35 \pm 1.08$  to  $105.27 \pm 0.82$  mg/g DW. The addition of different percentages of water could notably reduce the viscosity of DES and positively affect polar component extraction. Surpassing the 25 % water addition threshold resulted in decreased extraction yields of bioactive compounds within DES (ChCl-Gly-CA). This reduction can be attributed to the detrimental effect on interactions between analytes and DES. The excessive water addition could lead to the disruption of hydrogen bonds, diminishing molecule interactions progressively (Ali et al., 2019). Polar water forms hydrogen bonds with both HBA and HDB, disrupting the hydrogen bonds matrix between them, and destabilizing DES structures. Solvent's viscosity becomes excessive below 25 % water addition, causing uneven mixing with GP powder. The results indicate that 25 % water addition to DES fosters a more efficient bioactive compound extraction system.

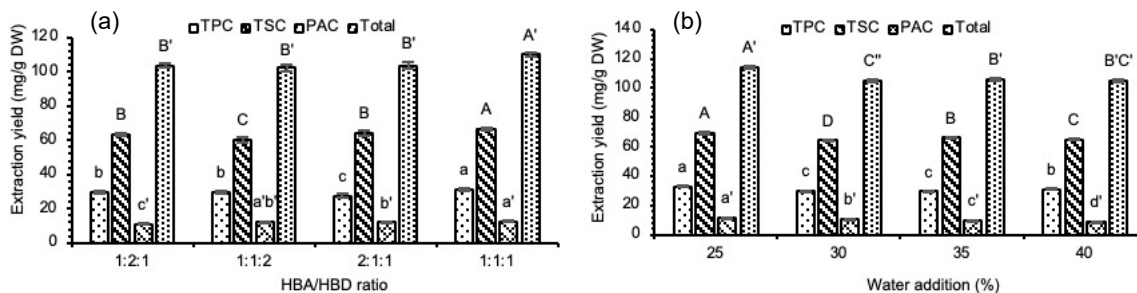


Figure 2: The impact of a) HBA:HBD ratio and b) water addition on the extraction yield of bioactive compounds

### 3.3 The effect of the solvent-to-material ratio and extraction temperature

The solvent-to-material ratio significantly impacted extraction yield and subsequent purification economics. The results showed that higher ratios initially favored phenolic compound extraction, yet beyond a certain point, excessive solvent diminished bioactive compound content (Figure 3a). Per mass transfer principle, more solvent increased bioactive compounds contact until equilibrium, beyond which extraction plateaued. In this study, bioactives increased until a 25:1 (v/w) ratio, then declined. TPC content insignificantly differed between the 20:1 and 25:1 (v/w) ratios. The TSC reached its maximum value ( $69.70 \pm 0.40$  mg AE/ g DW) at a 25:1 (v/w) ratio. Lower solvent-to-material ratios resulted in a lower extraction capacity of the DES, slowing the process. Higher solvent-to-material ratios increased the extraction capacity of the solvent, enabling shorter extraction times. Excessive ratios led to reduced bioactive compound content and solvent wastage. This aligns with a study of Lin et al. (2022), where escalating ratios from 25:1 to 30:1 lowered compound recovery efficiency. Optimizing ratios is crucial for efficient extraction and cost-effectiveness purification.

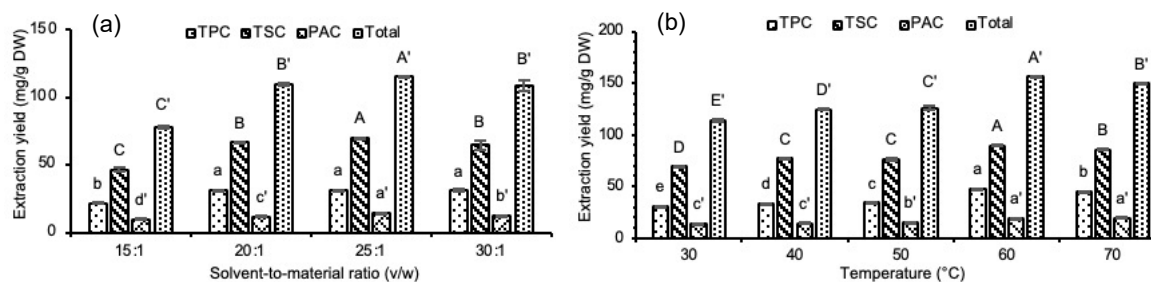


Figure 3: The influence of a) solvent-to-material ratio and b) extraction temperature on the extraction yield

Figure 3b shows TPC and TSC peaked at 60 °C and decreased with higher extraction temperature. At 30 °C, TPC, TSC, and PAC were  $30.48 \pm 0.28$  mg GAE/g DW,  $69.14 \pm 1.03$  mg AE/g DW,  $14.20 \pm 0.14$  mg CE/g DW. At 60 °C, peaks were TPC of  $47.49 \pm 0.23$  mg GAE/g DW, TSC of  $89.47 \pm 0.74$  mg AE/g DW, PAC of  $19.30 \pm 0.17$  mg CE/g DW. Beyond 60 °C, the trend declined to TPC  $44.82 \pm 0.42$  mg GAE/g DW, TSC  $85.36 \pm 0.62$  mg AE/g DW, and PAC  $19.50 \pm 0.42$  mg CE/g DW at 70 °C ( $p < 0.05$ ). Bioactive compounds are thermally labile, degrading at higher temperatures. Both extract biological activity and the extraction efficiency are significantly influenced by temperature. In DES extraction, temperature crucially determines solvent viscosity. Higher temperature enhances compound extraction due to improved solvent diffusivity within the matrix and greater solubility of phenolic compounds. Reduced solvent viscosity boosts diffusivity. Based on results and comparison, 60 °C emerged as the ideal temperature for GP bioactive compound extraction using by DES.

### 3.4 The effect of the extraction time

Bioactive compounds increased with extended extraction time, peaking at 2.5 h, then declining. Figure 4 illustrates TPC, TSC, and PAC rising from 25 % to 33 %. Prolonging extraction 3 h led to approximately 32 % decrease in total bioactive compound content. Solid-liquid extraction is a mass-transfer process involving substance migration from a solid matrix to a solvent through osmotic and diffusion mechanisms. Prolonged extraction ruptures more plant cells, increase the collected bioactive compounds. After 2.5 h, additional compounds plateau, as evident in Figure 4. Extending extraction exposes the phenolic compounds longer to light, oxygen, and temperature, promoting oxidation and degradation. The observed decline in compounds past 2.5 h likely results from this. An appropriate extraction time for maximal bioactive compound content seems around 2.5 h.

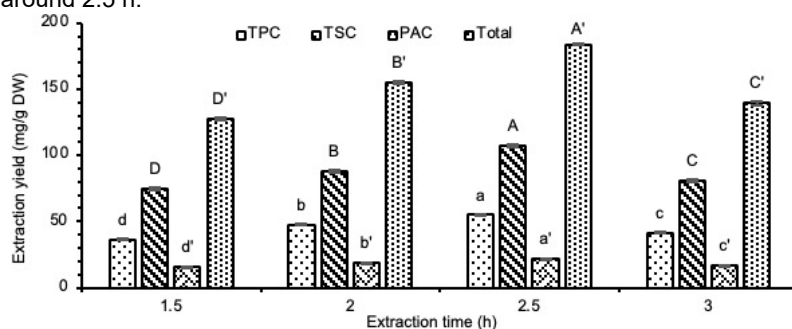


Figure 4: The impact of extraction time on the extraction yield of bioactive compounds

### 3.5 Comparison

The study determined the contents of various bioactive compounds, namely catechin, epicatechin, gallic acid, quercetin, kaempferol, myricetin, resveratrol, and rutin. To demonstrate the efficacy of DES extraction, a comparison was made between the content of bioactive compounds extracted using DES, ethanol, and methanol. The results, as shown in Table 1, clearly indicate that DES extraction under the best conditions in this study resulted in a noteworthy enhancement in the quantity of TPC, TSC, PAC, individual phenolic compounds compared to organic solvents. Using the same raw material for solvent comparison revealed that higher initial TPC, TSC, and PAC contents in the raw material led to increased extraction yield. Recovery efficiency results indicated insignificant differences under identical conditions. Further research is required to standardize starting material for consistent extraction yields across batches. Noteworthy is the finding that the extraction of catechin, epicatechin, rutin, kaempferol, myricetin, and quercetin was substantially higher when using DES, ranging from twice to eleven times the yield obtained from ethanol and methanol extraction solvents. The finding aligns with the study results of Dabetić et al. (2020), DES for higher extraction efficiency of bioactive compounds from grape skin compared with ethanol. Previous research has also shown that the presence of catechin and rutin in *Red Cardinal* grapes (Topalovic and Mikulic-Petkovsek, 2010).

Table 1: Comparison of bioactive compounds content extracted by different extraction solvents

Compound	DES	50 % Ethanol <sup>1</sup>	Ethanol Ref. <sup>2</sup>	Methanol Ref. <sup>3</sup>
TPC (mg GAE/g DW)	65.491 <sup>a</sup> ± 1.298	27.368 <sup>c</sup> ± 0.000	16.056 <sup>d</sup> ± 0.169	57.002 <sup>b</sup> ± 1.570
TSC (mg AE/g DW)	177.881 <sup>a</sup> ± 5.167	66.540 <sup>c</sup> ± 1.247	40.218 <sup>d</sup> ± 1.258	120.266 <sup>b</sup> ± 0.009
PAC (mg CE/g DW)	36.218 <sup>a</sup> ± 0.771	12.090 <sup>c</sup> ± 0.000	5.730 <sup>d</sup> ± 0.132	29.067 <sup>b</sup> ± 1.569
Catechin (mg/g DW)	0.144 <sup>a</sup> ± 0.003	0.041 <sup>c</sup> ± 0.007	0.026 <sup>d</sup> ± 0.001	0.074 <sup>b</sup> ± 0.000
Epicatechin (mg/g DW)	0.151 <sup>a</sup> ± 0.026	0.055 <sup>b</sup> ± 0.009	0.032 <sup>b</sup> ± 0.002	0.046 <sup>b</sup> ± 0.001
Gallic acid (mg/g DW)	0.005 <sup>c</sup> ± 0.000	0.011 <sup>a</sup> ± 0.000	0.008 <sup>b</sup> ± 0.000	0.011 <sup>a</sup> ± 0.001
Kaempferol (mg/g DW)	0.016 <sup>a</sup> ± 0.000	0.006 <sup>b</sup> ± 0.000	0.006 <sup>c</sup> ± 0.000	n.d.
Myricetin (mg/g DW)	0.221 <sup>a</sup> ± 0.183	0.034 <sup>a</sup> ± 0.005	0.021 <sup>a</sup> ± 0.001	0.106 <sup>a</sup> ± 0.000
Quercetin (mg/g DW)	0.020 <sup>b</sup> ± 0.000	0.008 <sup>c</sup> ± 0.000	0.008 <sup>c</sup> ± 0.000	0.061 <sup>a</sup> ± 0.000
Resveratrol (mg/g DW)	n.d.	0.035 <sup>a</sup> ± 0.015	0.015 <sup>a</sup> ± 0.000	n.d.
Rutin (mg/g DW)	0.142 <sup>a</sup> ± 0.057	0.122 <sup>a</sup> ± 0.059	0.067 <sup>a</sup> ± 0.017	0.140 <sup>a</sup> ± 0.002

<sup>1</sup>The best extraction conditions in this current study; <sup>2</sup>Extraction conditions according to method of Caldas et al. (2018); <sup>3</sup>Extraction conditions according to method of Iglesias-Carres et al. (2018); n.d.: Not detected; Distinct letters within the same row indicate statistically significant differences between extracts obtained by different solvents ( $p \leq 0.05$ ).

#### 4. Conclusions

This study demonstrated that the extraction conditions of DES, including DES type, HBA/HBD ratio, water addition, solid-to-solvent ratio, temperature, and time, significantly influenced bioactive compound content. The most suitable DES extraction parameters were identified as ChCl: Gly: CA (1:1:1, w/w/w), 25 % water addition, a 25:1 solvent-to-material ratio, and extraction at 60 °C for 2.5 h. Under these conditions, the TPC, TSC, and PAC values were measured as  $65.49 \pm 1.30$  mg GAE/g DW,  $177.88 \pm 5.17$  mg AE/g DW,  $36.22 \pm 0.77$  mg CE/g DW. The results demonstrate the superiority of DES over traditional chemical solvents in enhancing bioactive compound extraction, particularly individual polyphenols. Exploring innovative techniques, such as combining DES with ultrasound, may further improve extraction yield from GP. The potential enhancement offers prospects for more efficient extraction and broader applications in various industries, promoting sustainable GP utilization.

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