Effect of acidity/alkalinity of deep eutectic solvents on the extraction profiles of phenolics and biomolecules in defatted rice bran extracts

Piraporn Sombutsuwan[1], Erwann Durand[2,3*], Kornkanok Aryusuk[1,4*]

[1] Lipid Technology Research Laboratory, Pilot Plant Development and Training Institute (PDTI), King Mongkutìs University of Technology Thonburi (KMUTT), Bangkok, Thailand.
[2] CIRAD, UMR QualiSud, F-34398, France.
[3] Qualisud, Univ Montpellier, Avignon UniversitÎ, CIRAD, Institut Agro, IRD, UniversitÎ de la RÎunion, Montpellier, France.
[4] Division of Biochemical Technology, School of Bioresources and Technology, King Mongkutìs University of Technology Thonburi (KMUTT), Bangkok, Thailand.

Corresponding Authors:
Kornkanok Aryusuk[1,4]: 49 Soi Thian Thale 25, Bang Khun Thian Chai Thale Road, Tha Kham, Bang Khun Thian, Bangkok 10150, Thailand. E-mail: kornkanok.ary@kmutt.ac.th
Erwann Durand[2,3]: CIRAD, UMR Qualisud, 73 Rue Jean FranÈois Breton, 34398 Montpellier, France. E-mail: erwann.durand@cirad.fr

Abstract

This study investigated the impact of acidity/alkalinity of deep eutectic solvents (DES) on the extraction profiles of phenolics and other biomolecules (phytic acid, reducing sugar, and protein) in defatted rice bran (DFRB). The DES with different pH levels were prepared by varying the hydrogen bond acceptor (ChCl and K_2CO_3) and hydrogen bond donor (lactic acid, urea, and glycerol). The results indicated that the acidic DES (ChCl-lactic acid; pH 0.42) exhibited excellent extraction efficiency for total phenolic acids (4.33 mg/g), phytic acid (50.30 mg/g), and reducing sugar (57.05 mg/g) while having the lowest protein content (5.96 mg/g). The alkaline DES (K_2CO_3-glycerol; pH 11.21) showed the highest levels of total phenolic acid (5.49 mg/g) and protein content (12.81 mg/g), with lower quantities of phytic acid (1.04 mg/g) and reducing sugar (2.28 mg/g). The weakly acidic DES (ChCl-glycerol; pH 4.72) exhibited a predominant total phenolics (3.46 mg/g) with lower content of protein (6.22 mg/g), reducing sugar (1.68 mg/g) and phytic acid (0.20 mg/g). The weak alkaline DES (ChCl-urea; pH 8.41) resulted in lower extraction yields for total phenolics (2.81 mg/g), protein (7.45 mg/g), phytic acid (0.10 mg/g), and reducing sugar (7.36 mg/g).

Additionally, the study delved into the distribution of phenolics among various DESs employed, revealing that the alkaline DES (K_2CO_3-glycerol) exhibited the highest concentration of free phenolics. Notably, ChCl-based DESs contained predominantly soluble esterified bound phenolics and soluble glycosylated bound phenolics. Moreover, a significant correlation was observed between antioxidant activities and phenolic contents.

In conclusion, this study explored the effect of acidity/alkalinity of DES on the extraction of phenolics and other value-added biomolecules in DFRB. The findings highlight the potential for manipulating the properties of DES through pH variation, making them versatile solvents for extracting and isolating valuable compounds from agricultural by-products such as DFRB and offering opportunities for sustainable utilization and value addition in various industries.

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Introduction

Rice bran, a coproduct derived from the milling process of husked rice (*Oryza sativa L.*), constitutes approximately 10 wt% of the rice grain (Gul et al., 2015). Remarkably, rice bran plays a crucial role in the food industry, serving as a valuable source for rice bran oil, contributing to a global market valued at $1.23 billion in 2018. This surging demand for rice bran oil has led to the substantial production of defatted rice bran (DFRB), a primary byproduct of the oil extraction process (Alexandri et al., 2020). Despite the inherent nutritional richness and presence of bioactive compounds, the utilization of DFRB remains predominantly limited to low-value applications such as animal feed or disposal in landfills (Alexandri et al., 2019; Gul et al., 2015). It is important to note that DFRB contains bioactive phenolics, which have exhibited a range of properties, including antioxidative effects (Zhao et al., 2018), anti-inflammatory effects (Yin et al., 2019), and the potential for preventing chronic conditions like cancer (Dokkaew et al., 2019) and cardiovascular diseases (Zhang et al., 2020).

In addition, DFRB contains a notable content of myo-inositol-1,2,3,4,5,6-hexakisphosphoric acid (IP6), commonly known as phytic acid (ranging from 5.90% to 6.48% on a dry basis). This compound has been associated with various health advantages, including antimicrobial effects (Nassar et al., 2021), antioxidative properties (Canan et al., 2021), and the potential to prevent colon cancer (Kaur et al., 2020). DFRB is a rich source of protein with distinctive attributes, encompassing emulsion, foaming, gel-forming, and hypoallergenic properties (Zhuang et al., 2019). Moreover, there have been reports on the conversion of DFRB into reducing sugars through fermentation for applications in both food and bioenergy sectors (Alexandri et al., 2020). Incorporating DFRB into value-added products offers opportunities to increase market value and develop innovative strategies that can influence the broader economic landscape.

However, the bioactive compounds within DFRB are predominantly bound to the cell wall, existing as insoluble macromolecules or within cell wall components like cellulose and structural proteins (Zhao et al., 2018). The release of bioactive compounds and biomolecules from the cell wall, followed by their dissolution in solvents, represents an important and critical step in obtaining extracts enriched with value-added biomolecules. While conventional methods involve strong alkaline or strong acid treatments for release (Shahidi & Hossain, 2023) encounter challenges due to their corrosive nature and potential environmental impacts. These issues are at odds with the prevailing emphasis on green chemistry and sustainable processes.

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Emerging as a promising alternative, deep eutectic solvents (DES) have gained attention as a novel class of solvents (Abbott et al., 2003). DES comprise associations between a halide salt or hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) (Zhang et al., 2012), and they offer advantages in terms of cost-effectiveness, ease of preparation, minimal energy requirements, biodegradability and toxicity (Yang, 2019; Zhang et al., 2012). DES have been extensively studied for the extraction of bioactive and biomolecules from agricultural wastes, including phenolic compounds (PC) (Jablonsky et al., 2020; Ruesgas-Ramˆ n et al., 2020; Santos

et al., 2021), proteins (Olalere & Gan, 2023) and the pretreatment of lignin and cellulose (Mnasri et al., 2023; Zhu et al., 2023).

The pH of DES on the extraction process has been recognized as a key factor influencing efficacy due to its substantial impact on the interaction between the DES and the target solute, particularly with respect to cell wall breakdown. Certain DES mixtures, such as ChCl:La and K:Gly, have demonstrated enhanced efficiency in dissolving biopolymers compared to DES with near-neutral pH (Tan Ngoh & Chua, 2018). Furthermore, both acidic (ChCl:La) and alkaline (K:Gly) DES have effectively facilitated the delignification of various plant residues (SuopajÊrvi et al., 2020). Based on this theory, the cell wall of defatted rice bran would have a high possibility of breaking down and releasing the bioactive molecules.

Previous studies have primarily concentrated on the output, compositions, and bioactive attributes of free phenolic compounds (FPC) or individual value-added biomolecules. This has created a gap in our understanding of how DES impact profiles, quantities, and functional qualities, including esterified-bound and glycosylated-bound PC. These forms of PC are solubilized and interact with macromolecules through covalent, ester, ether, or CóC bonds (Wang et al., 2020), and they have gained growing attention due to their health-promoting attributes in food (Arruda et al., 2018; Wang et al., 2022; Zhong et al., 2022).

Therefore, the present study aims to investigate how the acidity and alkalinity of DES influence the phenolic and biomolecule compositions within extracts from DFRB. Furthermore, the investigation seeks to uncover distinct phenolic profiles along with their corresponding potential as antioxidants. These efforts offer valuable insights into the promising application of these compounds as ingredients for promoting health and well-being.

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Materials & Methods

2.1 Chemicals

Phenolic acid standards, including 4-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, ferulic acid, syringic acid, sinapic acid, and caffeic acid, along with phytic acid, bovine serum albumin, and glucose, were obtained from Sigma-Aldrich (St. Louis, MO, USA). The additional chemicals such as potassium persulfate ($K_2S_2O_8$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), choline chloride (ChCl), Folin Ciocalteu reagent, glycerol, lactic acid, and potassium carbonate (K_2CO_3) were also obtained from Sigma-Aldrich (Saint Louis, USA). Ethyl acetate, hexane, and acetic acid of HPLC grade were sourced from RCI Lab Scan Co. Ltd., Bangkok, Thailand. All other chemicals were of analytical grade and utilized without further purification.

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2.2 Preparation of DES

Four type DES compositions (detailed in **Table 1**) were formulated by mixing, stirring, and heating the constituents (hydrogen bond acceptor and hydrogen bond donor) in Erlenmeyer

flasks. The resulting mixture was continuously stirred and heated at 70±C until a clear and homogeneous liquid phase was achieved.

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2.3 DES properties

The pH of freshly prepared DES samples containing 20% (w/v) water was measured using a Mettler pH meter. The solvatochromic characteristics were evaluated employing Nile Red dye. Nile Red was introduced into each DES as a solvatochromic probe, and the □max value was determined to identify the wavelength of maximum visible light absorption as described previously (Laokuldilok et al., 2011; Mulia Fauzia & Krisanti, 2019). The DES-dye mixtures were scanned within the 400ó700 nm range using a UV-vis spectrophotometer. The Nile Red polar parameter (E_{NR}) was then calculated using Equation (1):

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$$E_{NR}\ (\text{kcal/mol}) = 28{,}591 / \lambda_{abs.max}\ (\text{nm}) \qquad (1)$$

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2.4 Extraction procedure

2.4.1 Plant material

Defatted rice bran (DFRB) was obtained from Surin Rice Bran Oil Co., Ltd. (Surin Province, Thailand). Proximate analysis of DFRB were determined according to Association of Official Analytical Chemists guidelines (AOAC, 2005) and the Van Soest sequential analytical method (Van Soest Robertson & Lewis, 1991).

2.4.2 Extraction of biomolecules in DFRB using DES

An outline depicting the process of DFRB extraction, and the characterization of phenolic compounds (PC) and value-added biomolecules is illustrated in **Figure 1**. To initiate the extraction, 2.5 g of DFRB were combined with 25 g of each DES in an Erlenmeyer flask. The mixture was then subjected to agitation in a water bath shaker at 150 rpm and a temperature of 70±C for 5 h. To reduce viscosity, distilled water (20%, w/w) was added into the sample. After completion of the extraction, the sample underwent centrifugation at 3500 rpm for 15 min., resulting in the collection of the supernatant. This supernatant was appropriately adjusted and subsequently stored at -20±C for further analysis of PC and biomolecules.

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2.5 Phenolic compounds determinations

2.5.1 Identification of phenolic compound types

The types of PC in the DES supernatant extracts were established based on the work of Lou et al. (Lou et al., 2020) with some modification as detailed below.

Free Phenolic Compounds (FPC)

The DES supernatant extracts were acidified to a pH of 2 using 6 mol/L HCl. The resulting supernatant underwent three successive extractions with ethyl acetate (EtAc) at a 1:1 (v/v) ratio. The EtAc phases were pooled, dehydrated using anhydrous Na_2SO_4, filtered, and subsequently dried with N_2 gas. The resultant dry residues were reconstituted in 5 mL of ethanol to yield the fraction containing free PC (FPC).

Soluble Esterified Bound Phenolic Compounds (SEBPC)

After the extraction of FPCs, the residual aqueous phase underwent hydrolysis with 4 M NaOH, containing 10 mM EDTA and 1% ascorbic acid for 4 h~~.~~ at room temperature. The sample was then acidified to a pH of 2 using 6 M HCl. The PC liberated from soluble esters were subjected to triple extractions using EtAc, following a similar procedure as employed for the FPCs, thus yielding the fraction of soluble esterified bound phenolic acid compounds (SBEPC).

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Soluble Glycosylated Bound Phenolic Compounds (SGBPC)

The residual aqueous phase after the extraction of SEBPC was subjected to additional hydrolysis using 6 M HCl at 75±C for 60 min. The PC released from soluble-bound glycosides were subsequently extracted three times using EtAc, following a similar methodology as employed for the extraction of FPCs. This process yielded the fraction containing soluble glycosylated bound phenolic acid compounds (SGBPC).

2.5.2 Determination of Total Phenolic Content (TPC)

The total phenolic content of the extracted fractions, namely FPCs, SBEPCs, and SBGPCs in DFRB, was determined utilizing the Folin-Ciocalteu method described by Kim & Lim (2016) with some modification. Twenty microliters of the sample were combined with freshly prepared Folin-Ciocalteu reagent (80 ∂l) and 7.5% (w/v) Na_2CO_3 (200 ∂l). The resulting mixture was diluted with 700 ∂l of distilled water and then placed in a dark environment at room temperature for 2 h. to allow the reaction to proceed. The assessment of total phenolic content was carried out using a microplate reader set to a wavelength of 765 nm (Thermo Fisher Scientific, US), using gallic acid as a standard. The quantity of total PC present in the sample was computed as gallic acid equivalents.

2.5.3 Characterization of phenolic acids using HPLC

Samples of FPC, SEBPC, and SGBPC derived from various types of DES were subjected to analysis of their phenolic acid profiles using high-performance liquid chromatography (HPLC). The HPLC system consisted of a pump model 515 (Water Associates, Milford, MA, USA), a Rheodyne 7125 six-port valve injector with a 10 ∂L loop, and a photodiode array detector (PDA; Shimadzu, Kyoto, Japan). The samples were prepared using the designated mobile phase and subsequently subjected to analysis on a Mightysil Si60 column (250Ÿ4.6 mm ID., 5 ∂m) protected with a Mightysil Si60 guard column (10Ÿ4.6 mm ID., 5∂m) (Kanto

Chemical Co. Inc., Tokyo, Japan). The mobile phase consisted of hexane/ethyl acetate/acetic acid (70:30:0.2, v/v/v) with a flow rate of 1.0 mL/min. UV absorbance was monitored in the 257-320 nm range (Sombutsuwan et al., 2021). HPLC control and data collection were performed using LC Solution Software (version 1.24, Shimadzu, Kyoto, Japan). Quantification of 4-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, ferulic acid, syringic acid, sinapic acid, and caffeic acid in the samples was performed using the external standard curve method.

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2.5.4 Anti-radical activity assays

DPPH radical-scavenging activity

The DPPH assay was determined according to Laokuldilok et al. (2011) with some modifications. Briefly, a 20 ∂l sample was mixed with 20 ∂l of ethanol, and the resulting mixture was added to 560 ∂l of 0.1 M DPPH in ethanol. This mixture was then incubated in darkness at 25±C for 30 min. Afterward, the absorbance at 517 nm was measured. The DPPH radical scavenging capacity of the sample was calculated using Equation (2)

$$\% \text{ DPPH radical-scavenging capacity} = [(A_c - A_s)/A_c] \text{ Ÿ } 100 \qquad (2)$$

~~where~~Where A_c represents the absorbance at 517 nm of the control, and A_s represents the absorbance at 517 nm of the sample. The DPPH radical scavenging capacity was expressed as mmol gallic acid/g DFRB.

ABTS assay

The methodology described previously (Sombutsuwan et al., 2021) was used for the determination of antioxidant capacity against ABTS with some modifications. Briefly, a stock solution comprising 7 mM ABTS solution and 2.45 mM potassium persulfate solution was prepared. After mixing the two stock solutions, the resulting solution was kept in the dark at room temperature for 12 h. The solution was then diluted with ethanol to achieve an absorbance of 0.7 ≤0.05 units at 734 nm using a spectrophotometer. A DFRB sample (20 ∂l) was mixed with 180 ∂l of $ABTS^{ñ+}$ solution and allowed to react for 6 min. The absorbance was measured at 734 nm using spectrophotometry. The $ABTS^{\bullet+}$ scavenging effect was calculated according to the Eq. (3):

$$ABTS^{\bullet+} \text{ radical scavenging activity } (\%) = [(A_c - A_s)/Ac] \text{ x } 100 \qquad (3)$$

where A_c was the absorbance at 734 nm of diluted $ABTS^{ñ+}$ solution at the beginning of the analysis and A_s was the absorbance at 734 nm of the mixture after 6 min. The ABTS radical scavenging capacity was expressed as mmol gallic acid/g DFRB.

2.6 Determination of value-added biomolecules

2.6.1 Phytic acid

Phytic acid content in the DES supernatant of DFRB extract was carried out using the Wade reagent method as described by Gao et al. (2007). A 5 mL aliquot of the DES extract supernatant was passed through a Dowex anion exchange column (0.25g). Inorganic phosphorous and interfering compounds were eluted with 7.5 ml of 0.1 M NaCl, followed by elution of phytate with 7.5 ml of 0.7 M NaCl. The eluted sample (3 mL) was mixed with 1 mL of the Wade reagent for 10 min. Measurement was conducted using a microplate reader at 500 nm. The DES solution served as the blank control. The phytic acid content in the samples was calculated based on a phytic acid standard curve.

2.6.2

The protein content within the DFRB extract, derived from the DES supernatant, was quantified using the Bradford assay (Bio-Rad Protein Assay Cat# 500-0006, Bio-Rad Laboratories Ltd., Thailand). The quantification procedure adhered to the manufacturer's guidelines. Briefly, 10 ∂l of the sample was mixed with 200 ∂l of Bradford reagent for 5 min. Subsequently, measurement was conducted utilizing a spectrophotometer set to 595 nm. The DES solution was employed as the blank control for reference. The protein content in the DFRB extract was calculated based on a Bovine serum albumin standard curve.

2.6.3 Reducing sugar

Reducing sugar content was determined using the dinitrosalicylic acid (DNS) assay (Miller, 1959). A 100 ∂l of DES supernatant of DFRB extract was mixed with 100 ∂l of DNS reagent. The mixture was incubated in a water bath at 80±C for 30 minutes. After cooling to room temperature, the sample was measured using a spectrophotometer at 575 nm. The DES solution was used as the blank control. The reduced sugar content in the DFRB extract was calculated based on a glucose standard curve.

3. Results

3.1 Analysis of the DFRB compositions and DES properties

Table 2 summarizes the proximate analysis of the DFRB, highlighting its primary components as hemicellulose (23.22%) and protein (18.00%), with minor amounts of ash, cellulose, and lignin.

The study also examined the pH and polarity of different DES. These DES types were categorized as strong acid (ChCl:La), weak acid (ChCl:Gly), weak alkaline (ChCl:U), and high alkaline (K:Gly) based on their pH properties. **Table 3** indicated slight polarity decreases among the DES types, measured by E_{NR} values where higher values indicated lower polarity. ChCl:La

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had the highest polarity (E_{NR} 47.73 kcal/mol), while other DES types showed slightly varying polarities.

3.2 Effect of DES type on the extraction of phenolic compounds and value-added biomolecules

Table 4 displays the concentrations and biomolecule types obtained from DFRB extraction using the different DES. The findings indicate that the high alkaline DES (K:Gly) yielded the highest concentrations of total PC (5.49 mg/g DFRB) and protein (12.81 mg/g DFRB) but lower levels of phytic acid (1.04 mg/g) and reducing sugar (2.28 mg/g DFRB). On the other hand, the strong acid DES (ChCl:La) yielded the highest concentrations of phytic acid and reducing sugar, along with a notable presence of total PC (4.33 mg/g DFRB). However, protein concentration from ChCl:La treatment was the lowest compared to other DES types. The weak acid DES (ChCl:Gly) and weak alkaline DES (ChCl:U) resulted in moderate protein concentrations (6.22 and 7.45 mg/g DFRB) and lower total PC compared to strong acid and high alkaline DES types.

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3.3 Effect of DES type on the selectivity of extraction of phenolic compounds

Figure 2 displays the contents and profiles of PC in various DES extracts, as analyzed using the Folin-Ciocalteu assay. The observed PC types include free form (FPC), soluble esterified bound (SEBPC), and soluble glycosylated bound (SGBPC). The K:Gly treatment showed the highest FPC at 1.81 mg/g DFRB. Moreover, the K:Gly treatment exhibited substantial SEBPC and SGBPC contents, approximately 1.67 mg/g DFRB and 2.27 mg/g DFRB, respectively. Distinct patterns were observed in the PC profiles between potassium (K)- and ChCl-based DES. In K:Gly treatment, the three PC types (FPC, SEBPC, and SGBPC) had similar contents. On the other hand, ChCl-based DES showed dominance of SEBPC and SGBPC, with lower amounts of FPC. For instance, in ChCl:La, ChCl:Gly, and ChCl:U treatments, SEBPC contents were 1.61, 1.30, and 1.01 mg/g DFRB, respectively, while SGBPC contents were 2.27, 1.91, and 1.60 mg/g DFRB, respectively. ChCl-based DES exhibited lower FPC contents, ranging from 0.19 to 0.46 mg/g DFRB.

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3.4 Compositions of phenolic compounds

The composition of phenolic acids within various PC fractions under different DES treatments was analyzed by HPLC. The primary phenolic acid identified across all DES types was ferulic acid, constituting a substantial portion (42.6 – 76.5%, w/w) of both free and bound PC (**Figure 3 (A)-(C)**). However, the different DES treatments yielded distinct compositions of PC in both free and bound forms. In particular, the FPC was predominantly composed of ferulic acid ranging from 42.5% to 76.5% and *p*-coumaric acid ranging from 22.4% to 54.1%. It is noteworthy that the FPC obtained from the K:Gly extraction exclusively exhibited a dominance of ferulic acid and *p*-coumaric acid at 76.21% and 22.63%, respectively. While, the FPC derived from ChCl:U extraction exhibited fairly similar amounts of ferulic acid and *p*-coumaric acid.

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Sinapic acid was significantly more abundant in bound form extracts (ranging from 11.37% in SGBPC extracted with K:Gly to 46.84% in SEBPC extracted with ChCl:Gly. Notably, SGBPC consistently exhibited a higher vanillic acid content compared to FPC and SEBPC fractions across most DES extractions. A similar trend was seen for 4-hydroxybenzoic acid, albeit with lower concentrations.

3.5 Antioxidant activities of free- and bound-form phenolic compounds

The potential antioxidant activities of free- and bound-form PC were evaluated using the DPPH and ABTS assays, as presented in **Table 5**. Similar patterns for radical scavenging activities were observed using both assays. Among the treatments, the PC derived from K:Gly treatments exhibited the highest overall activities, with values of 15.69 mmol (expressed as gallic acid equivalent per gram (GAE/g)) for DPPH and 27.99 mmol GAE/g for ABTS assays. Notably, the total antioxidant activities of PC extracted from ChCl-based DES were comparatively lower than those from potassium-based DES (K:Gly). The weakest antioxidant activities were found in PC obtained from ChCl:U treatment, measuring 1.96 mmol GAE/g for DPPH and 10.61 mmol GAE/g for ABTS assays. Analyzing the total PC contents resulting from various treatments, the values were 5.55 mg/g for K:Gly, 4.33 mg/g for ChCl:La, 3.46 mg/g for ChCl:G, and 2.81 mg/g for ChCl:U (refer to **Table 4**). The ranking of antioxidant activities determined by both assays followed the order: K:Gly > ChCl:La > ChCl:G > ChCl:U, consistent with the total PC contents.

4. Discussion

4.1 DFRB compositions and DES properties.

The primary constituents of DFRB were identified as hemicellulose and protein, aligning with previous research findings. According to Moreira et al. (2022), DFRB contained lignin, hemicelluloses, cellulose, ash, and moisture in percentages of 8.63%, 11.56%, 7.81%, 10.60%, and 12.4%, respectively. Additionally, the protein content in DFRB was reported to be 17% (Zhuang et al., 2019).

Based on pH measurements, the DES used in this study can be categorized into four groups: strong acid (ChCl:La), weak acid (ChCl:Gly), high alkali (K:Gly), and weak alkali (ChCl:U). Notably, the pH values of each DES type align closely with previous reports. The pH values of ChCl:La, ChCl:Gly, and ChCl:U are within the range of 0 to 1, as reported by Juri□et al. (2021), Ruesgas-Ramˆn et al. (2020) and Thi & Lee (2019), respectively. Furthermore, the pH value of K:Gly falls between 11 and 12, according to the previous study (Lim et al., 2019). According to the E_{NR} assessment, ChCl:La displayed the highest extinctive polarity within the studied range of DES. The polar characteristics of K:Gly closely paralleled those of ChCl:U and ChCl:Gly. Pandey & Pandey (2014) utilized solvatochromic probes to quantitatively measure the polarity of four different DES formulations. These DESs were prepared by combining ChCl with glycerol, urea, malic acid, and ethylene glycol in a 1:2 molar ratio. The outcomes of their investigation revealed that the notable polarity observed in these DESs mainly arose from the

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inherent hydrogen bond donor (HBD) characteristics present in their components. Furthermore, an investigation revealed that natural deep eutectic solvents (NADES) based on organic acids exhibited greater polarity than those consisting of alcohols and sugar-based components (Dai et al., 2013).

4.2 Effect of DES type on the extraction of phenolic compounds and value-added biomolecules

The effects of DES types on the extraction of total phenolic content and value-added biomolecules from DFRB were investigated and are shown in **Table 4**. Notably, DES variations exhibited distinct profiles of bioactive compounds derived from DFRB. The results revealed that the highly alkaline K:Gly demonstrated remarkable efficiency in extracting PC and protein, while it did not exhibit the same proficiency in extracting phytic acid and sugars. Conversely, the potent acid, ChCl:La, exhibited excellent extraction capabilities for phytic acid, reducing sugars, and phenolic content. ChCl:gly and ChCl:U, representing weak acid and weak alkaline types, respectively, showed moderate efficacy in extracting a range of bioactive compounds. It was observed that PC and other biomolecules were primarily bound within the cell wall of DFRB (Zhuang et al., 2019).

The pH of DES emerged as a key factor influencing the extraction efficiency of PC and value-added biomolecules. DES with low or high pH values exhibited the potential to disrupt plant cell walls more effectively, thereby facilitating the release of bioactive molecules into the DES solution. Tan et al. (2018) reported that DES types with harsh pH conditions, such as ChCl:La and K:Gly, demonstrated enhanced efficiency in dissolving biopolymers compared to DES with pH levels closer to neutral. Moreover, the acidic ChCl:La and alkaline K:Gly have been shown to effectively delignified wheat, corn, and rapeseed stem residues (SuopajÊrvi et al., 2020).

The higher phenolic contents were observed in ChCl:La and K:Gly, which were strong acid and strong alkaline DES, respectively. These conditions appeared to facilitate the degradation of the cell wall, leading to the release of PC. ChCl:La had been reported as an effective solvent for extracting PC from rice bran and other agricultural waste (Jablonsky et al., 2020; Ruesgas-Ramˆ n et al., 2020; Santos et al., 2021). Notably, the utilization of K:Gly treatment caused the disappearance of ^{13}C NMR spectra signals of PC in residual pomace (Loow et al., 2016). Furthermore, the breakdown of polysaccharides into monomers, resulting in reducing sugars, was linked to the cleavage of glycosidic linkages under acidic conditions. Corn stover treated with ChCl:La demonstrated the presence of reducing sugars (Liang et al., 2021). In addition, the higher quantity of reducing sugars in the liquid fraction of ChCl:La-treated samples was obtained compared to ChCl:Gly, ChCl:U, and K:Gly treatments (Tan et al., 2021).

The alkaline extraction method is commonly used to obtain proteins from plant materials due to its simplicity and affordability. These conditions had been found to increase protein extraction yield and solubility of the extracted protein (Juul et al., 2023; Phongthai Lim & Rawdkuen, 2016). In this study, the highest protein content was obtained from K:Gly treatment

(**Table 4**), aligning with a previous study that employed K:Gly as medium for the extraction of protein from defatted wheat germ (Olalere & Gan, 2023). In contrast, phytic acid displayed notable solubility in acidic conditions, leading to its dissociation from proteins or mixed salts (Canan et al., 2011; Saad et al., 2011). As a result, the pronounced acidic characteristics of ChCl:La played a significant role in driving the elevated phytic acid content observed in this research.

4.3 Effect of DES type on the selectivity of extraction of phenolic compounds and the resulting potential in antioxidant activities

In this section, we investigate the influence of different types of DES on phenolic composition and anti-radical activities. It is worth highlighting that the K:Gly treatment demonstrated the highest concentration of FPC. Additionally, among the ChCl-based DES, ChCl:La stood out for its significant presence of soluble-bound PC (**Figure 2**).

The impact of pH on the liberation of bioactive compounds was discussed in Section 4.2. It was evident that relatively weaker acidic and basic conditions yielded a reduced release of bioactive compounds, likely due to a less efficient degradation of the cell wall, consequently affecting the total release of PC. Phenolic compounds bound to the cell wall components (such as cellulose, hemicellulose, pectin, lignin, and structural proteins) were found to be more abundant than their free counterparts (Harukaze Murata & Homma, 1999; Zhou et al., 2004).

Alkaline hydrolysis demonstrated greater effectiveness in liberating PC from their bound state in comparison to acid hydrolysis (Nenadis Kyriakoudi & Tsimidou, 2013; Vadivel & Brindha, 2015). The soluble-bound forms, consisting of SEBPC and SGBPC, were observable in both acid and alkaline DES treatments. This could be attributed to the capability of DES to dissolve and modify constituents of the cell wall, such as lignin, cellulose, hemicellulose, and protein, thereby leading to their degradation into smaller molecules. It has been reported that acidic DES possess the ability to cleave lignin-carbohydrate linkages and lignin ether bonds, whereas alkaline DES have shown a more pronounced effect on lignin removal compared to hemicellulose (Guo et al., 2022). Additionally, studies have documented the successful extraction of oligosaccharide hydroxycinnamates from wild rice through the application of acid hydrolysis (Bunzel et al., 2002), as well as the extraction of feruloylated arabinoxylans from nixtamalized maize bran achieved via alkaline hydrolysis (Herrera-Balandrano et al., 2020).

Ferulic acid and *p*-coumaric acid emerged as prominent PC in DFRB. These compounds were detected across all types of DES and in all forms of PC (as illustrated in **Figure 3**). Furthermore, additional minor PC, including *p*-hydroxybenzoic acid, vanillic acid, and sinapic acid, were also identified. These observations are in line with a study by Kim et al. (2006), who identified *p*-hydroxybenzoic and vanillic acids in both free and bound forms of red and white wheat bran. Additionally, Qiu Liu & Beta (2010) identified monomeric phenolic acids in wild rice, including *p*-coumaric, vanillic, syringic, and *p*-hydroxybenzoic acids, as well as phenolic acid aldehydes, which were found in both soluble and insoluble forms. Similar outcomes were reported by Laokuldilok et al. (2011), who highlighted the prevalence of ferulic acid in rice bran,

accompanied by lower quantities of gallic, protocatechuic, hydroxybenzoic, *p*-coumaric, and sinapic acids in the bound form.

Furthermore, the PC extracted using different types of DES displayed distinct variations in radical scavenging activities, as indicated in **Table 4** and **Table 5**. A clear correlation was observed between the overall efficiency and the total phenolic contents. Notably, the ABTS assay revealed a stronger trend in antioxidant activity when compared to the DPPH assay. This difference can be attributed to varying mechanisms, primarily involving hydrogen atom transfer (to scavenge free radicals through hydrogen donation) and electron transfer (to reduce compounds by transferring electrons) (Apak, 2019).

5. Conclusions

This study investigates the influence of acidity and alkalinity within DES on the extraction of PC and other value-added biomolecules from DFRB. The outcomes highlight the potential of adjusting DES properties by controlling pH levels, thus making them adaptable solvents that effectively extract and isolate valuable compounds from agricultural by-products such as DFRB. These findings open up promising opportunities for sustainable utilization and value enhancement across various industries. Further works are ongoing to implement a strategy where pH-tuned DES may be used to drive the optimum composition of the natural extract, especially with the aim of promoting its application as an antioxidant ingredient.

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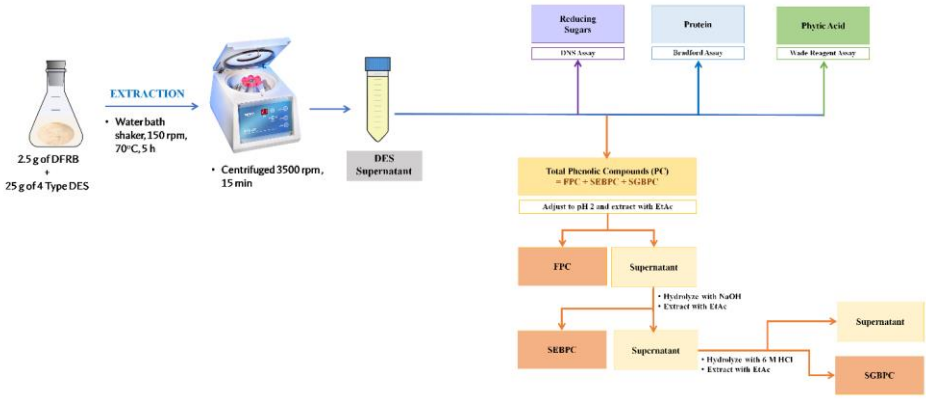
Figure legends

Figure 1. Overview of the DFRB extraction process, along with the characterization of phenolic compounds and value-added biomolecules.

Figure 2. Impact of DES type on phenolic compounds contribution: FPC (free phenolic compounds), SEBPC (soluble esterified bound phenolic compounds), SGBPC (soluble glycosylated bound phenolic compounds), and TPC (total phenolic compounds).
Values with the same letter in the same PC types are not significantly different ($p < 0.05$).

Figure 3. Compositions of phenolic compounds extracted from DFRB using different types of DES: ChCl:La (choline chloride-:Lactic acid), ChCl:Gly (choline chloride:Glycerol), ChCl:U (choline chloride:Urea), and K:Gly (potassium carbonate:Glycerol).



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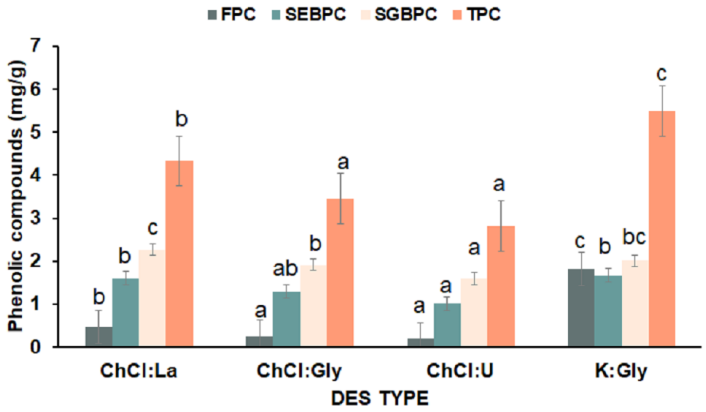

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Comentado [MR16]: Not clear

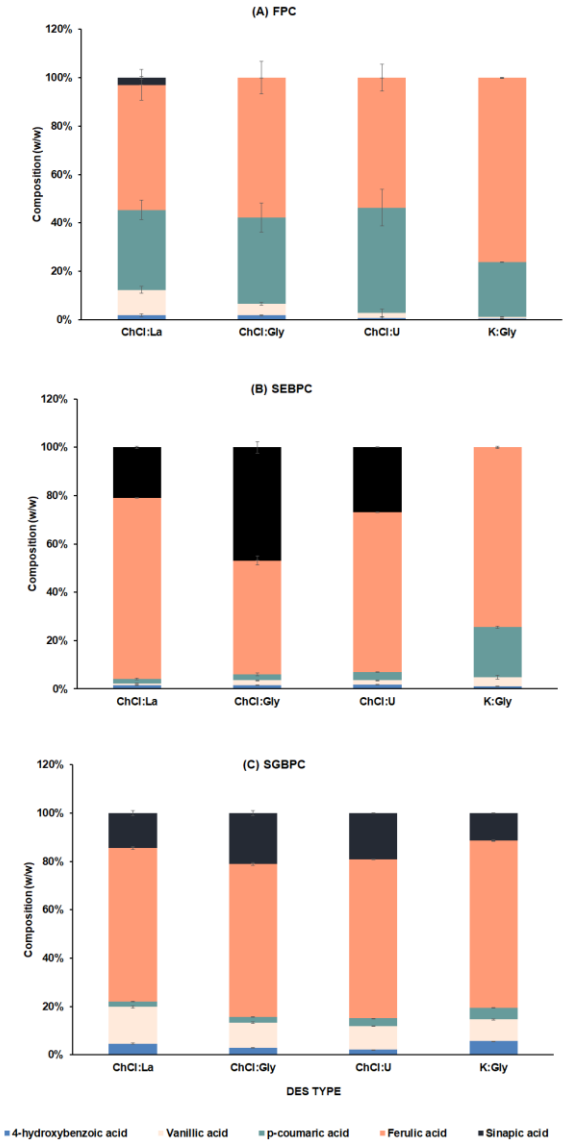

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Table 1 Composition of DES.

Hydrogen bond acceptor	Hydrogen bound donor	Molar ratio	Abbreviations
Choline chloride	Lactic acid	1:2	ChCl:La
Choline chloride	Glycerol	1:2	ChCl:Gly
Choline chloride	Urea	1:2	ChCl:U
Potassium carbonate	Glycerol	1:7	K:Gly

Comentado [MR17]: Water amount?

Table 2 Proximate analysis of defatted rice bran (DFRB).

Composition	Amount (% w/w)
Protein	18.00 ± 0.00
Hemicellulose	23.22 ± 0.66
Cellulose	8.20 ± 0.05
Lignin	4.27 ± 0.18
Moisture	5.11 ± 0.01
Ash	11.74 ± 0.04

Comentado [MR18]: Why w/w?

Table 3 Properties of DES.

DES	pH	E_{NR} (kcal/mol)
ChCl:La	0.42	47.73
ChCl:Gly	4.72	49.04
ChCl:U	8.41	49.64
K:Gly	11.21	49.98

Table 4 Effect of DES on total phenolic content and value-added biomolecules from DFRB.

DES	Content (mg/ g DFRB)			
	Total phenolic compounds	**Proteins**	**Phytic acids**	**Reducing sugars**
ChCl:La	$4.33 \leq 0.33^{b}$	$5.96 \leq 0.12^{a}$	$50.30 \leq 5.23^{b}$	$57.05 \leq 2.87^{c}$
ChCl:Gly	$3.46 \leq 0.23^{a}$	$6.22 \leq 0.31^{a}$	$0.20 \leq 0.07^{a}$	$1.68 \leq 0.21^{a}$
ChCl:U	$2.81 \leq 0.20^{a}$	$7.45 \leq 0.29^{b}$	$0.10 \leq 0.03^{a}$	$7.36 \leq 0.21^{b}$
K:Gly	$5.49 \leq 0.33^{c}$	$12.81 \leq 0.15^{c}$	$1.14 \leq 0.02^{a}$	$2.28 \leq 0.08^{a}$

Comentado [MR19]: Why they are in red?

Table 5 Antioxidant activities of DES extracts of free and bound form phenolic compounds evaluated using the DPPH and ABTS assays.

Anti-radical activity (mmol GAE/g)								
DES	**DPPH**				**ABTS**			
	FPC	SEBPC	SGBPC	Total	FPC	SEBPC	SGBPC	Total
ChCl:La	0.57 ≤ 0.17	2.66 ≤ 0.45	4.71 ≤ 0.12	7.94 ≤ 0.24	1.09 ≤ 0.03	3.53 ≤ 1.02	17.91 ≤ 2.00	22.57 ≤ 1.49
ChCl:Gly	0.11 ≤ 0.06	1.08 ≤ 0.43	3.63 ≤ 0.53	4.83 ≤ 0.86	1.16 ≤ 0.20	8.41 ≤ 2.03	9.72 ≤ 1.32	19.29 ≤ 1.54
ChCl:U	0.09 ≤ 0.04	0.52 ≤ 0.53	1.96 ≤ 0.29	2.57 ≤ 0.70	1.17 ≤ 0.23	4.01 ≤ 1.07	5.42 ≤ 0.36	10.60 ≤ 1.38
K:Gly	4.02 ≤ 0.37	4.77 ≤ 0.87	6.90 ≤ 0.87	15.69 ≤ 1.43	12.47 ≤ 0.99	6.58 ≤ 1.51	8.95 ≤ 1.22	27.99 ≤ 0.87