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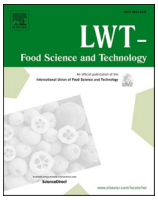

Greener extraction of anthocyanins and antioxidant activity from blackberry (*Rubus* spp) using natural deep eutectic solvents

Oscar Zannou *, Ilkay Koca

Department of Food Engineering, Ondokuz Mayis University, 55139, Samsun, Turkey

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ABSTRACT

Natural deep eutectic solvents (NaDESs) are new green solvents used for the extraction of phenolic compounds. In this work, an efficient and greener strategy for extracting blackberry anthocyanins using NADESs was investigated. Sixteen NADESs constituted of choline chloride-urea, choline chloride-sugars, choline chloride-organic acids, choline chloride-polyalcohols and organic acids-sugars were synthesized. The antioxidant properties and anthocyanin profile of blackberry extracts obtained with NADESs and conventional solvents (water, methanol and ethanol)were evaluated. Results of the characterization of NADESs showed that the viscosity, pH, electric conductivity and chemical structure of NADESs were greatly influenced by the hydrogen bond acceptors and hydrogen bond donors. The highest recovery efficiencies were recorded with NADESs giving the highest total phenolic content (9.35 ± 0.39 mg GAE/G), total flavonoid content (104.72 ± 5.17 mg ECE/100g), total anthocyanin content (115.37 ± 0.43), antiradical activity (68.77 ± 2.29 mmol TE/g), reducing power (83.08 ± 3.78) and metal chelating activity (4771.25 ± 83.58 mg TE/100g). NADESs were the most prominent solvents for the recovery of the targeted anthocyanins, with the more acidic NADESs extracting higher quantities of cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin chloride and pelargonidin-3-glucoside. A greener extraction using NADES can therefore effectively and reliably targets antioxidants and particularly anthocyanins in blackberry, offering promising ways for future investigations on the phytochemicals in plants.

1. Introduction

New sources of healthy foods containing bioactive compounds are being investigated as adequate solutions for health and nutrition. The bioactive compounds can help prevent many diseases, including cancers, coughing, inflammatory, cardiovascular and oxidative stress diseases (Pappas & Schaich, 2009). The phenolic compounds are one of the most popular bioactive compounds that have a wide range of biological activities and are widely used in food, cosmetic and pharmaceutical industries. The extraction of the phenolic compounds from vegetal materials has got urge attention of these last decades. Generally, for the industrial-scale extraction of these bioactive compounds, conventional organic solvents such as hexane, chloroform, alcohols and ethyl acetate are used. However, the use of large quantities of these organic solvents has the drawbacks of having undesirable effects on the environment and leaving unacceptable residues in the extracts (Puranik, Sanjay Pai, & Rao, 2009). In this context, the development of green solvents has emerged as an alternative to producing environmentally friendly solvents that have high extraction efficiency (Zhang et al., 2012; Barbieri et al., 2020).

Natural deep eutectic solvents (NADESs) became one the most prominent green solvents regarding their eco-friendly aspects and extraction efficiencies (Bi et al., 2020; Zannou, Koca, Aldawoud, & Galanakis, 2020). NADESs are formulated with natural hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA). These components form a network of hydrogen bonds that results in a lower melting point, a strong supramolecular structure, biodegradable, non-toxic, renewable, and affordable (El Kantar et al., 2019; Zannou & Koca, 2020). NADESs result from the combination of vegetal cellular constituents such as choline derivatives, sugars, amino acids or organic acids. Their application for the extraction of phenolic compounds from various vegetal materials has shown successful and probative results (Loarce, Oliver-Simancas, Marchante, Díaz-Maroto, & Alañón, 2021; Vázquez-González et al., 2020). They are one of the most convenient means for the extraction of phenolic compounds due to their higher extractability efficiency compared to conventional organic solvents (Wan Mahmood, Lorwirachsutee, Theodoropoulos, & Gonzalez-Miquel, 2019; Zannou et al., 2020). Moreover, NADESs display high

* Corresponding author. Department of Food Engineering, Faculty of Engineering, Ondokuz Mayis University, 55139, Samsun, Turkey.
E-mail address: zannouoscar@gmail.com (O. Zannou).

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solubilization power and stabilizing ability, making them perfect media for the extraction of bioactive compounds and for the sustainable valorization of vegetal materials (Fernández, Boiteux, Espino, Gomez, & Silva, 2018; Wei et al., 2015).

Rubus spp., commonly known as blackberry, is one of the most spread species of Rosaceae. The fruit is traded for its prized taste, pleasant flavor and nutritional potentials. The fruit is consumed fresh or processed into jam, wine, tea, ice cream, desserts, seedless jellies and bakery products. The extract obtained from fruits are used as natural colorants in food industry (Kaume, Howard, & Devareddy, 2011; Zia-Ul-Haq, Riaz, De Feo, Jaafar, & Moga, 2014). Blackberry fruit is a rich source of protein, carbohydrates, ascorbic acid, sugar, carotenoids and minerals as well as vitamins (Zia-Ul-Haq, Riaz, De Feo, Jaafar, & Moga, 2014). A wide spectrum of phytochemical compounds has been reported in blackberries, including phenolic acids, flavonoids, tocopherols, carotenoids and organic acids (Moraes et al., 2020; Tomas et al., 2019). The subgroup anthocyanin of flavonoids is amongst the key components in blackberries (Da Fonseca Machado et al., 2018; Yamashita et al., 2017). The main anthocyanins reported in blackberries consisted of cyanidins including cyanidin-3-glucoside, followed by cyanidin-3-xyloside, cyanidin-3-malonylglucoside, cyanidin-3-dioxalylg lucoside and cyanidin-3-sambubioside (Zia-Ul-Haq et al., 2014). The consumption of blackberry promotes health since it exhibited many biological activities due to its richness in bioactive compounds (Hussein et al., 2018; Moraes et al., 2020; Tomas et al., 2019).

To the best of the authors' knowledge, no published studies investigating the feasibility of NaDESs to extract the phenolic compounds from blackberry have been found in the existing literature. The aim of the present study was to develop and establish a convenient, efficient and eco-friendly method for the extraction of anthocyanins from blackberry by using NADESs combined with ultrasound-assisted extraction. Sixteen different NADESs consisting of natural hydrogen bond acceptors and hydrogens bond donors were investigated. Distilled water, methanol and ethanol were used as conventional solvents for comparison. The antioxidant properties of blackberry extracts in terms of their total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), antioxidant radical scavenging (DPPH), ferric reducing power (FRAP) and iron chelating activity (FCA) were evaluated. In addition, the extractability of individual anthocyanins was investigated.

2. Material and methods

2.1. Raw material

Mature blackberry was purchased from the local market in Samsun, Turkey. The fruits were sorted and pressed to obtained the pulp. The pulp has a soluble solids content of 11.50 ± 0.50 brix, pH of 3.00 ± 0.03, dry matter of 11.80 ± 0.19% and 1.46 ± 0.12% malic acid. The pulp was roughly homogenized, about 50 g of pulp were taken into tubes and stored at −40 °C until the experiments.

2.2. Chemical and reagents

Folin–Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol (HPLC grade), ethanol (HPLC grade), acetonitrile (HPLC grade), sodium carbonate, sodium acetate, sodium nitrite, sodium hydroxide, hydrochloric acid (37%), choline chloride (≥98%), D-sorbitol (97%), xylitol (99%), malic acid (≥98%), 1,3-butanediol (≥99.5%), ferrozine, iron chloride, and standards were provided from Sigma Aldrich Chemical Co. (St Louis, MO, USA). Gallic acid, L(+) tartaric acid (>99%), ethylene glycol (≥99.5%), glacial acetic acid (≥99.5%) and iron sulfate were purchased from Carlo Erba (Emmendingen, Germany). Lactic acid (90%), citric acid (≥99.7%), D(+)-glucose (≥97.5%) and urea (≥99%) were taken from Isolab (Eschau, Germany). Aluminum chloride and iron chloride were acquired from Merck while glycerol (≥99.5%) from Tekkim (Nilufer, Bursa).

Table 1
The compositions, molar ratio and acronyms of NADESs.

No	Component 1	Component 2	Molar ratio	Water content (%)	Acronyms
1	Choline chloride	Urea	1:2	20	CHUR
2	Choline chloride	Glucose	1:2	20	CHGLU
3	Choline chloride	Xylitol	1:2	20	CHXYL
4	Choline chloride	Lactic acid	1:2	20	CHLA
5	Choline chloride	Acetic acid	1:2	20	CHAC
6	Choline chloride	Citric acid	1:2	20	CHCI
7	Choline chloride	Glycerol	1:2	20	CHGLY
8	Choline chloride	Butanediol	1:2	20	CHBUT
9	Choline chloride	Ethylene glycol	1:2	20	CHETHGLY
10	Lactic Acid	Sorbitol	1:2	20	LASOR
11	Acetic acid	Sorbitol	1:2	20	ACSOR
12	Tartaric acid	Xylitol	1:2	20	TARXYL
13	Tartaric acid	Sorbitol	1:2	20	TARSOR
14	Citric acid	Xylitol	1:2	20	CIXYL
15	Malic acid	Xylitol	1:2	20	MAXYL
16	Malic acid	Sorbitol	1:2	20	MASOR

2.3. NADESs preparation

The NADESs were prepared following the preparation protocol described previously by Chanioti and Tzia (2018). Sixteen NADESs were prepared at 1:2 M ratio of HBA and HBD with the addition of 20% of distilled water (Table 1).

2.4. Rheology

The viscosities of NADESs were measured at 20 °C with a Rheometer (Buchi, CH-9230 Flawil 1, Switzerland) fitted with a parallel geometry with 20 mm of diameter and gap 1 mm (Zannou & Koca, 2020).

2.5. pH analysis

pH was measured using a pH-meter (Model Starter 3100, OHAUS, NJ, USA).

2.6. Fourier transformed infrared (FTIR) analysis

FTIR analysis of NADESs and extracts was carried out at the wavenumbers of 4000 and 400 cm^{-1} using a FTIR Spectrometer (Perkin Elmer, Spectrum-Two, USA, PEService 35) (Zannou et al., 2020).

2.7. Conductivity

Electrical conductivity properties were measured using an electrochemical analyzer (Consort c6010, Turnhout, Belgium). The measurements were performed at 25 °C and the values were recorded as μS. cm^{-1}.

2.8. Extraction

The extraction was carried out with distilled water, methanol and ethanol (conventional solvents) and NADESs in a sonication water bath (WUCA03H, ultrasonic cleaner set, daihan scientific Co., Ltd, Wonju-Shi, South Korea). Before the experiments, the frozen pulp was thawed

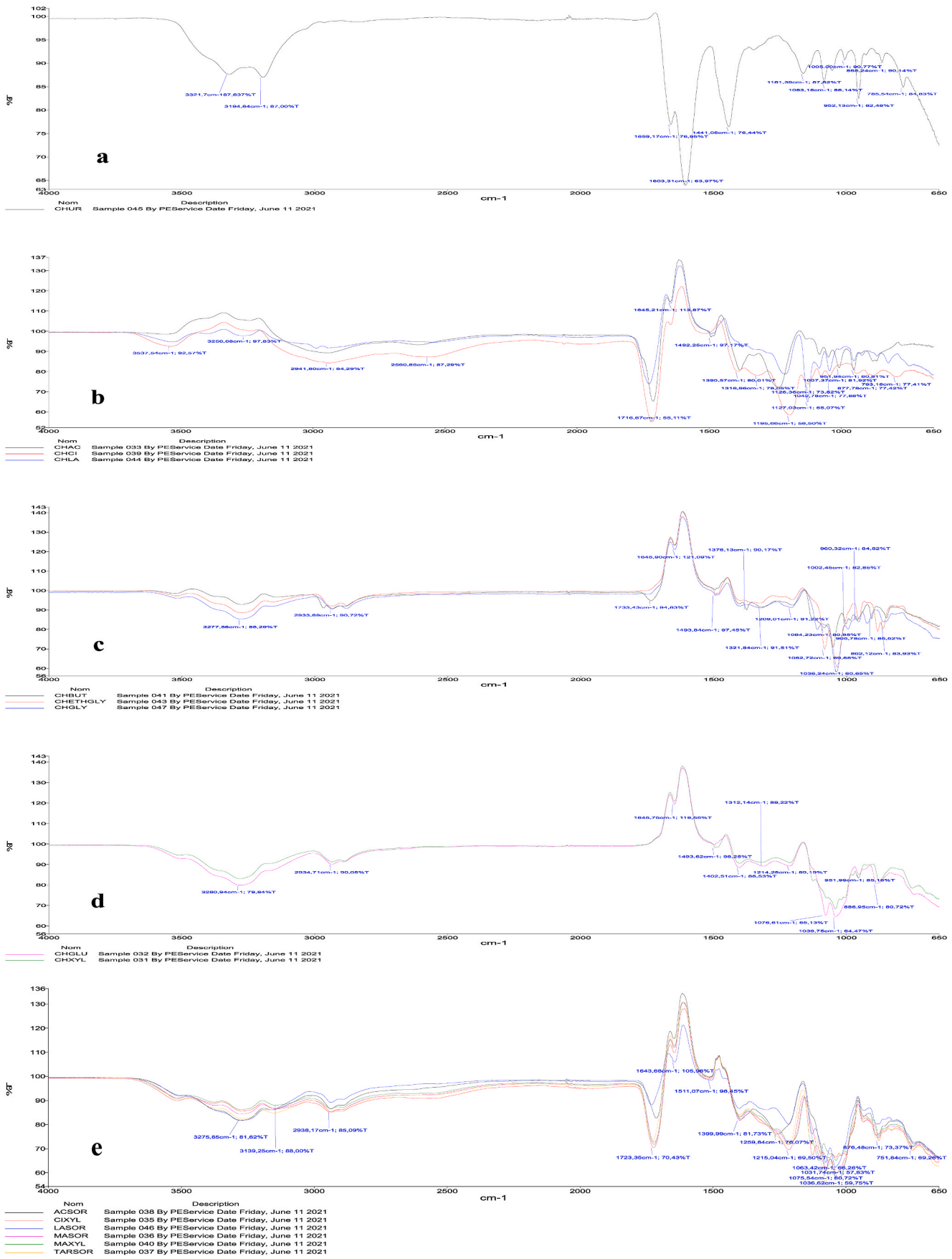

Fig. 1. FTIR spectra of NADESs; a) choline chloride-urea, b) choline chloride-acids, c) choline chloride-alcohols, d) choline chloride-sugars and e) acids-sugars.

at room temperature and 0.5 g was mixed with 10 mL of conventional or NADES solvents. The mixture was ultrasonicated at 25 °C for 20 min. The samples were then filtered through Whatman filter paper No.1 thrice.

2.9. Total phenolic content (TPC)

TPC was evaluated by Folin-Ciocalteu method adopted from Singleton and Rossi (1965) with some modifications. Briefly, 150 μL of samples were mixed with 750 μL of 10% Folin-Ciocalteu reagent (5 min) and 600 μl of 7.5% Na_2CO_3. The mixture was kept in dark for 2 h and the absorbance was read at 760 nm. TPC was expressed as mg gallic acid equivalent per g (mg GAE/g).

2.10. Total flavonoid content (TFC)

TFC was determined adopting the procedure mentioned in Zannou and Koca (2020). The absorbance was read at 510 nm. The results were given as mg epicatechin equivalent per g (mg ECE/g).

2.11. Total anthocyanin content (TAC)

TAC was determined with the pH differential method reported in Lee, Durst, Wrolstad, and Collaborators (2005). The absorbances of the samples containing pH 1 and pH 4.5 were read at 510 and 700 nm. TAC was expressed as mg cyanidin-3-glucoside equivalent per 100g (mg CGE/100g).

2.12. Determination of antioxidant activity

2.12.1. DPPH radical scavenging activity assay (DPPH)

DPPH assay was conducted following the method of Odabaş and Koca (2020). The absorbance was read against a control. The values of DPPH radical scavenging were determined with a calibration curve as mmol Trolox equivalent per g (mmol TE/g).

2.12.2. Ferric reducing antioxidant power assay (FRAP)

FRAP assay was conducted following the method of Benzie and Strain (1996). The value of FRAP was obtained from a standard curve of $FeSO_4$. The results were given as mmol $FeSO_4$ equivalents per g (mmol ISE/g).

2.12.3. Fe^{2+} chelating activity assay (FCA)

The chelating activity was measured according to the method previously described (Dinis, Madeira, & Almeida, 1994) and Santos, Brizola, and Granato (2017) with some modifications. Briefly, to 0.5 mL of adequately diluted extract, 1.6 mL of distilled water and 0.05 mL of $FeCl_2$ (2 mM) were added. After 30 s, 0.1 mL of ferrozine (5 mM) was added. After 30 min at room temperature, the absorbance was read at 562 nm. The percentage of Fe2+ -ferrozine complex formation was calculated as: Fe^{2+} chelating rate (%) = [($Abs_{solution\ containing\ extract,\ FeCl2\ and\ ferrozine}$ - $Abs_{solution\ without\ ferrozine}$)/$Abs_{control}$] x 100 (Santos et al., 2017). The values of FCA were determined with a calibration curve as mg Trolox equivalent per g (mg TE/g).

2.13. Determination of individual anthocyanins

The individual anthocyanins were identified using the previous method of Bosiljkov et al. (2017) with modifications. The anthocyanins were determined using a high-pressure liquid chromatography (HPLC) system (Agilent 1260; Agilent Technologies) with a diode array detector (DAD) at 520 nm wavelength. The anthocyanins were separated in an Inertsil ODS-4 column (3 μm, 4,6 × 50 mm; GL Sciences Kat No: 5020–0404) at a 1 mL min^{-1} flow rate. The mobile phases were: (A) 94% 2 mM sodium acetate and 6% acetic acid (v/v); and (B) acetonitrile. The following elution gradient was used, according to solvent B: 0–20min, 14%–23%; 20–40min, 23%–35%; 40–50min, 40%; 50–60min, 60%; 60–65min 95%. The column temperature was set at 30 °C. The individual anthocyanins were identified by comparing their retention times with their respective standard. The identified anthocyanins were quantified using a mixture of external standards (cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin chloride and pelargonidin-3-glucoside) which were prepared at different concentrations.

Table 2
Physicochemical characteristics of NADESs.

No:	NADESs	Characteristics of NADESs		
		Viscosity (mPa)	pH	Conductivity ($\mu S.cm^{-1}$)
1	CHUR	14.35 ± 0.07	9.99 ± 0.01	1114.33 ± 53.54
2	CHGLU	1093.00 ± 4.58	5.30 ± 0.07	307.00 ± 8.54
3	CHXYL	585.33 ± 3.79	5.05 ± 0.01	190.80 ± 4.00
4	CHLA	9.36 ± 0.08	0.76 ± 0.01	1135.67 ± 71.60
5	CHAC	8.75 ± 0.09	1.32 ± 0.01	1059.33 ± 16.20
6	CHCI	168.33 ± 1.15	−0.46 ± 0.01	138.00 ± 3.61
7	CHGLY	23.48 ± 0.12	5.36 ± 0.02	777.00 ± 13.75
8	CHBUT	0.39 ± 0.05	5.99 ± 0.08	695.00 ± 3.46
9	CHETHGLY	7.55 ± 0.37	7.04 ± 0.01	1141.67 ± 53.41
10	LASOR	347.67 ± 0.58	1.81 ± 0.02	3.50 ± 0.05
11	ACSOR	466.67 ± 0.58	2.20 ± 0.03	0.88 ± 0.00
12	TARXYL	771.33 ± 0.58	1.03 ± 0.01	9.86 ± 0.15
13	TARSOR	2063.67 ± 1.53	1.20 ± 0.08	6.57 ± 0.03
14	CIXYL	244.00 ± 0.00	1.12 ± 0.02	15.90 ± 0.06
15	MAXYL	484.33 ± 0.58	1.31 ± 0.01	4.98 ± 0.03
16	MASOR	742.00 ± 1.00	1.43 ± 0.02	3.63 ± 0.02

2.14. Statistical analyses

All results were expressed as the mean of three replicates ±standard deviation. Statistical analyses were done using a one-way analysis of variance ANOVA, and the significance of the difference between means was evaluated by Duncan's multiple range test. Differences at $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Characteristics of NADESs

3.1.1. FTIR spectra

The FTIR spectra of choline chloride-urea, choline chloride-organic acids, choline chloride-sugars and organic acids-sugars NADESs are showed in Fig. 1a, b, c, d and e, respectively. As can be seen in Fig. 1, NADESs showed low intensive of OH stretching bands at wavenumber 3500-3200 cm^{-1}, suggesting that they contain a very low water content (Ghaedi, Ayoub, Sufian, Lal, & Uemura, 2017; Da Silva et al., 2020). Only 20% of water was added to the NADESs to tailor their viscosity and to facilitate their application. The OH stretching vibration of NADESs at the wavenumber of 3300–3100 cm^{-1} indicated the formation of hydrogen bonding between HBA and HBD (Ghaedi et al., 2017; Ozturk, Parkinson, & Gonzalez-Miquel, 2018) and confirmed the synthesis of NADES solvents. In Fig. 1a, the wavenumber at 3455 cm^{-1} can also be referred to N–H stretching frequency of urea (Manivannan & Rajendran, 2011). The wavenumber at 3200-2932 cm^{-1} referred to C–H stretching bands. In Fig. 1b and e, the peak at 1723 cm^{-1} wavenumber is associated to COO of carboxylic acids, confirming the presence of the organic acid in the mixtures. The peaks obtained in all NADESs around 1645 cm^{-1} wavenumber are related to C═C stretching vibrations. In Fig. 1a, the intense peak observed at 1603 cm^{-1} can also be referred to N–H deformation frequency of urea (Manivannan & Rajendran, 2011). Moreover, the peaks obtained at the 1500-600 cm^{-1} region were grouped in the C–O, CH, C–C and OCO stretching, deformation or bending vibrations of NADESs (Fu et al., 2021).

3.1.2. Viscosity and pH

The viscosity is one of the most essential characteristics of NADESs. It is an important obstacle for the NADESs application. The highly viscous NADES hinders the mass transfer, affecting adversely the recovery of bioactive compounds (Wei et al., 2015). The viscosity of the NADESs varied immensely depending on their composition ($p < 0.05$) (Table 2). The viscosity of the studied NADESs was in the range of 0.39–2063.67 mPa. The choline chloride-sugar or organic-sugar-based NADESs exhibited the highest viscosity while choline-organic acid or choline chloride-alcohol-based exhibited the lowest values. Thus, TARSOR exhibited the highest viscosity, followed by CHGLU, TARXYL, MASOR, CHXYL, MAXYL, ACSOR, LASOR and CISOR. The high viscosity of these NADESs can be attributed to the formation of hydrogen bonds that results in loss of molecular mobility. The higher viscous characters of the NADESs can also be attributed to the longer chain length of their chemical structure (Gajardo-Parra, Cotroneo-Figueroa, Aravena, Vesovic, & Canales, 2020). Moreover, the high viscosity of the NADESs can be due to the molar ratio of the HBA and HBD. The molar ratio should be appropriately chosen to avoid obtaining very viscous or saturated NADES.

The pH behavior of the sixteen NADESs was evaluated at room temperature and the results were given in Table 2. As can be seen, the components used for the formation of NADESs influenced directly the pH of the final solvents. This observation was found in accordance with the results of several studies where NADESs were formed with different components (Chen et al., 2019; Zannou & Koca, 2020). The pH of CHUR was the highest (9.99 ± 0.01), which is supported by Adeyemi, Abu-Zahra, and AlNashef (2018) who have found the pH of alkanolamine-choline chloride deep eutectic solvents ranging between 10 and 13. Choline chloride-sugar, choline chloride-organic acid and choline chloride-alcohol-based NADESs presented the pH of 2.29 ± 0.02–5.05 ± 0.01, 0.76 ± 0.01–1.32 ± 0.01 and, 5.36 ± 0.02–7.04 ± 0.01, respectively. In NADESs where the organic acids were combined with sorbitol or xylitol, the pH value was found between 1.03 ± 0.01 and 2.20 ± 0.03. Evidently, the acid-based solvents presented the lowest pH, followed by the sugar-based and the alcohol-based NADESs. García, Rodríguez-Juan, Rodríguez-Gutiérrez, Rios, and Fernández-Bolaños (2016) have found pH values of 2 and 0.5 for choline chloride-lactic acid (1:2) and choline chloride-malonic acid (1:1) NADESs, respectively.

3.1.3. Conductivity

The conductivity shows how well a material can conduct electrical current, how a material can be resistive for the motion of electrons within its molecules and exerts the electrolyte properties. The electric conductivity of the investigated solvents was given in Table 2. NADESs in which choline chloride was used as HBA provided the highest electric conductivity ranging from 138.00 ± 3.61 to 1141.67 ± 53.41 μS cm^{-1}. The polyalcohol-choline had the highest electric conductivity (695.00 ± 3.46–1141.67 ± 53.41 μS cm^{-1}), followed by CHUR (1114.33 ± 53.54 μS cm^{-1}), acid-choline chloride (138.00 ± 3.61–1135.67 ± 71.60 μS cm^{-1}), sugar-choline chloride (190.80 ± 4.00–307.00 ± 8.54 μS cm^{-1}), respectively. In contrast, NADESs in which the organic acids were used as HBA had the lowest electric conductivity ranging from 0.88 ± 0.00 to 15.90 ± 0.06 μS cm^{-1}. This difference may be due to the ionic form of choline chloride (Dai, Witkamp, Verpoorte, & Choi, 2015). Thus, it may be concluded that the NADESs with choline chloride had a high electric conductivity while the NADESs with organic acid had low electric conductivity. These findings were in the same agreement with the results of Dai et al. (2015) have reported that the conductivity of NADESs decreases in the sequence of base-polyalcohol > base-organic acid ≈ base-sugar > organic acid-amino acid > organic acid-sugar > sugar--sugar. The conductivity values found in the present study seemed to correlate with the values of viscosity since the most viscous NADESs displayed low conductivity. Our results are supported by the literature where the same tendencies have been observed in many NADESs (Dai, van Spronsen, Witkamp, Verpoorte, & Choi, 2013; Siongco, Leron, Caparanga, & Li, 2013).

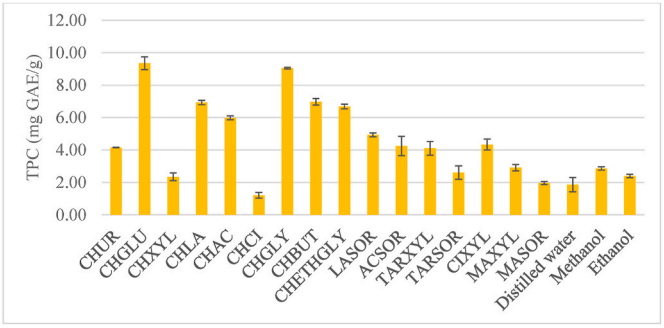

Fig. 2. Total phenolic content (TPC) recovery efficency of NADESs and conventional solvents.

3.2. Extractibility of bioactive compounds

3.2.1. Total phenolic extraction

The results showing the extraction performances of NADESs and conventional solvents were given in Fig. 2. NADESs provided TPC values ranging from 1.20 ± 0.17 to 9.35 ± 0.39 mg GAE/g while the water, methanol and ethanol gave TPC of 1.87 ± 0.44, 2.86 ± 0.10 and 2.35 ± 0.11 mg GAE/g, respectively. The NADESs exhibited higher TPC than water, methanol and ethanol, except CHCI, TARSOR, MASOR, MAXYL which showed slightly lower yields. The high extractability performances of the NADESs could be linked to the addition of 20% distilled water to tailor their viscosity. The hydrophilic NADESs have been reported to be more appropriate solvents for the extraction of phenolic compounds than the conventional solvents (El Kantar et al., 2019; Zannou et al., 2020). The high extractability performance of NADESs could be also associated to the presence of multiple hydrogen bonding networks which strongly interact with phenolic compounds, facilitating their extraction (Bakirtzi, Triantafyllidou, & Makris, 2016; Lakka et al., 2019). Dai et al. (2015) have reported that the extractability efficiency of NADESs is due to the formation of a strong intramolecular structure with the solute. The low extractibility efficiency of CHCI, TARSOR, MASOR, MAXYL could be obviously due to their high viscosity. Dai, Rozema, Verpoorte, and Choi (2016) have mentioned that the high viscosity of NADESs hinders the diffusion rate and mass transfer due to the extensive hydrogen-bonding network between HBA and HBD.

Comparing the TPC extraction efficiency of NADESs, choline chloride-based NADESs exhibited the best recovery yields (Fig. 2). Six NADESs showed the best performance including CHGLU (9.35 ± 0.39 mg GAE/g) and CHGLY (9.04 ± 0.05 mg GAE/g) which provided the highest TPC extraction efficiencies, followed by CHBUT (6.98 ± 0.20 mg GAE/g), CHLA (6.93 ± 0.14 mg GAE/g), CHEthGLY (6.68 ± 0.15 mg GAE/g) and CHAC (5.98 ± 0.12 mg GAE/g), respectively. The high performance of these NADESs could be due to the hydrophilicity of the phenolic compounds which increases the polarity of choline chloride and sugars, polyalcohols and organic acids. The intensity of the interaction between the NADESs and phenolic compounds is associated to the availability of hydrogen bonding (eg. OH and Cl). Consequently, the NADESs with a higher ability to interact and to form hydrogen bonding can be more efficient than others even though they own higher viscosity (Zhang, De Oliveira Vigier, K., Royer, & Jerome, 2012; Alsaud, Shahbaz, & Farid, 2021) (eg. CHGLU). The NADESs formed from the paire choline chloride and glucose ($C_6H_{12}O_6$), glycerol ($C_3H_8O_3$), butanediol ($C_4H_{10}O_2$), lactic acid ($C_3H_6O_3$), ethylene glycol ($C_2H_6O_2$) or acetic acid ($C_2H_4O_2$) seem to have the most ability to constitute the multiple hydrogen bonding networks with polar molecules such as phenolic compounds. These findings are in the same agrement with some previous studies which have reported that the NADESs obtained from choline chloride and glucose, glycerol, butanediol, lactic acid, ethylene glycol or

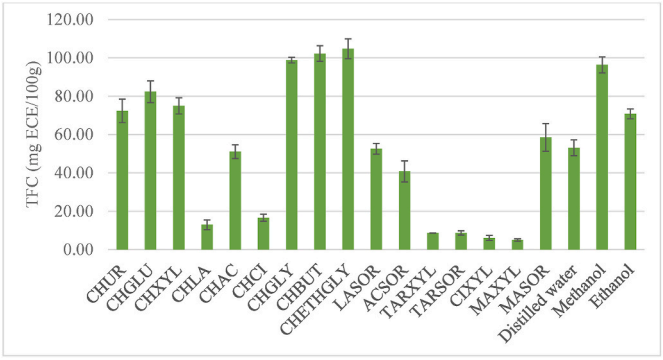

Fig. 3. Total flavonoid content (TFC) recovery efficency of NADESs and conventional solvents.

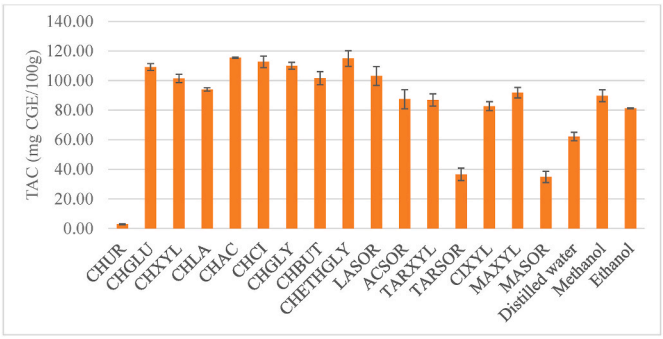

Fig. 4. Total anthocyanin content (TAC) recovery efficency via NADESs and conventional solvents.

acetic acid are prominent for the extraction of phenolic compounds (Wei et al., 2015; Wan Mahmood et al., 2019; Alsaud et al., 2021; de Almeida Pontes, Shiwaku, Maximo, & Caldas Batista, 2021).

3.2.2. Total flavonoid extraction

The recovery of the total flavonoids containing blackberry was evaluated with NADESs and compared to the conventional solvents such as distilled water, methanol and ethanol. The results were given in Fig. 3 and as one can see flavonoids' extraction performance of solvents varied significantly ($p < 0.05$). NADESs provided TFC values ranged from 4.95 ± 0.64 to 104.72 ± 5.17 mg ECE/100g while the water, methanol and ethanol gave 53.02 ± 4.11, 96.35 ± 4.20 and 72.30 ± 2.56 mg ECE/100g, respectively. NADESs CHETHGLY (104.72 ± 5.17 mg ECE/100g), CHBUT (102.26 ± 4.07 mg ECE/100g) and CHGLY (98.81 ± 1.48 mg ECE/100g) were found more efficient than water, methanol and ethanol. These results may be explained by the multiple hydrogen-bonding networks of these NADESs with flavonoids and the effect of solubility (Cheng, Yu, & Xue, 2020; Yu et al., 2020). Although the NADESs CHETHGLY, CHBUT and CHGLY exhibited the highest performances, MAXYL exhibited the lowest TFC. Clearly, the extraction potential of the tested NADESs is greatly influenced by HBD and HBA. On one hand, choline chloride and alcohol-based NADESs extracted more total flavonoid, followed by choline chloride-sugar and choline chloride-carboxylic acid-based NADESs, respectively. On the other hand, the carboxylic acid-sugar-based NADESs provided lower efficiencies. These results are in the same agreement with the findings of Shang et al. (2018) who have mentioned that the amine-alcohol-based NADESs gave higher performances in terms of flavonoid extraction than those of the sugar-carboxylic acid-based.

3.2.3. Total anthocyanin extraction

The total anthocyanin contents (TAC) of samples were shown in Fig. 4. The total monomeric anthocyanin contents recovered by NADESs were ranged from 2.82 ± 0.37 to 115.37 ± 0.43 mg CGE/100g, whereas the aqueous, methanolic and ethanolic extracts have given 62.14 ± 2.81, 89.73 ± 3.93 and 81.27 ± 0.32 mg CGE/100g, respectively. NADESs exhibited higher or similar extractability of anthocyanins compared to the conventional solvents, except CHUR, MASOR and TARSOR. The lowest TAC (2.82 ± 0.37 mg CGE/100g) was determined with CHUR due to its high pH (9.99 ± 0.01). It has been reported that the monomeric anthocyanins change the structure at increasing pH values and are degraded at pH below 7 (Cvjetko Bubalo, Ćurko, Tomašević, KovačevićGanić, & RadojčićRedovniković, 2016; Bosiljkov et al., 2017). Although MASOR and TARSOR are organic acid-based NADESs with pH below 2, they had high viscosities. The high viscosity is a drawback for the extraction efficiency of anthocyanin since it hinders the mass transfer rate (Dai et al., 2013; Zannou et al., 2020). Furthermore, Bosiljkov et al. (2017) have mentioned that the high viscosities can be a restrictive factor for using NADESs as extraction solvents due to the lower extraction performances and the energy required for stirring and pumping. The highest values of anthocyanins were found with CHAC (115.37 ± 0.43 mg CGE/100g), CHETHGLY (114.88 ± 5.29 mg CGE/100g), CHCI (112.67 ± 3.90 mg CGE/100g), CHGLY (110.04 ± 2.34 mg CGE/100g), CHGLU (109.06 ± 2.32 mg CGE/100g), LASOR (103.10 ± 6.42 mg CGE/100g), CHBUT (101.56 ± 4.44 mg CGE/100g), CHXYL (101.49 ± 2.78 mg CGE/100g). Generally, organic acid-based NADESs showed the highest efficiencies for the recovery of blackberry anthocyanin. These findings are supported by the previous studies which have assessed the effectiveness of the acidic NADESs to extract higher quantities of anthocyanins and polymeric pigments (Bi et al., 2020; Bosiljkov et al., 2017; Fu et al., 2021). Moreover, alcohol-based NADESs (CHETHGLY and CHGLY) extracted also higher quantities of blackberry anthocyanins due to their strong hydrogen bonding networks, polarity and relatively low viscosity. The highest TPC, TFC and TAC were determined in CHGLU, CHETHGLY and CHAC, respectively. This variation of NADESs for the extraction of bioactive compounds highlighted the affinity of NADESs for the recovery of the targeted analyte.

Table 3
Antioxidant characteristics of blackberry extracts obtained from NADESs and conventional solvents.

No	Solvents	Antioxidant activity		
		DPPH, mmol TE/g	FRAP (mmol ISE/g)	FCA (mg TE/100g)
1	CHUR	16.96 ± 0.92gh	15.36 ± 1.24i	5.13 ± 0.31i
2	CHGLU	35.78 ± 3.43e	32.13 ± 3.02d	10.48 ± 0.42hi
3	CHXYL	40.47 ± 9.14de	33.24 ± 3.19d	19.26 ± 2.51hi
4	CHLA	50.22 ± 2.77c	51.95 ± 2.15c	964.76 ± 41.79e
5	CHAC	53.02 ± 2.30b	53.47 ± 2.061c	341.51 ± 0.00g
6	CHCI	68.77 ± 2.29a	23.90 ± 0.86gh	1.97 ± 0.21i
7	CHGLY	48.21 ± 1.90bc	28.65 ± 0.65def	43.59 ± 0.00hi
8	CHBUT	40.26 ± 3.14de	24.23 ± 2.35fgh	0.19 ± 0.01i
9	CHETHGLY	25.71 ± 2.52f	29.38 ± 1.57de	20.93 ± 1.67hi
10	LASOR	29.20 ± 1.51f	52.05 ± 1.21c	508.67 ± 0.00f
11	ACSOR	43.56 ± 6.92cd	73.95 ± 4.36b	4687.67 ± 83.58a
12	TARXYL	18.00 ± 0.50gh	83.08 ± 3.78a	1.99 ± 0.10i
13	TARSOR	29.26 ± 6.32f	5.79 ± 0.57j	3684.71 ± 83.58b
14	CIXYL	26.37 ± 3.94f	13.10 ± 0.47i	1762.37 ± 83.58c
15	MAXYL	13.84 ± 1.39h	6.20 ± 0.40j	94.21 ± 2.09hi
16	MASOR	15.81 ± 1.69h	8.67 ± 0.89j	123.46 ± 2.09h
17	Distilled water	16.47 ± 0.30gh	20.22 ± 1.02h	0.04 ± 0.00i
18	Methanol	23.10 ± 2.32 fg	26.61 ± 2.01efg	1595.21 ± 83.58d
19	Ethanol	17.47 ± 0.40gh	23.40 ± 1.14gh	21.35 ± 2.93hi

a-i = Different letters in the same column indicate significant differences between solvents ($P < 0.05$); n.d. = Not detected.

3.3. Evaluation of antioxidant activity

3.3.1. DPPH radical scavenging activity (DPPH)

The DPPH method is used to study the ability of the antioxidants in an extract to scavenge free radicals in the solution or to donate hydrogen atoms, depending on the conditions. The results of the DPPH radical scavenging activity of samples were shown in Table 3. As can be seen, the DPPH of samples varied significantly ($p < 0.05$) depending on the types of solvents. The extracts obtained from NADESs exhibited higher antiradical activity compared to the conventional solvents, except the extracts obtained from CHUR, MAXYL and MASOR. Similarly, several studies have reported higher antiradical activity of extracts obtained from various plant materials using similar NADESs when compared to aqueous, methanolic and ethanolic extracts (Alsaud et al., 2021; Bakirtzi et al., 2016; Oliveira et al., 2021). The extracts obtained from NADESs provided the antiradical activity ranged from 13.84 ± 1.39 to 68.77 ± 2.29 mmol TE/g and the extracts obtained from the conventional solvents gave 16.47 ± 0.30, 23.10 ± 2.32 and 17.47 ± 0.40 mmol TE/g for distilled water, methanol and ethanol, respectively. The high antiradical activity was determined in the samples obtained from CHCI (68.77 ± 2.29 mmol TE/g), CHAC (53.02 ± 2.30 mmol TE/g), CHLA (50.22 ± 2.77 mmol TE/g), CHGLY (48.21 ± 1.90 mmol TE/g), ACSOR (43.56 ± 6.92), CHXYL (40.47 ± 9.14 mmol TE/g) and CHBUT (40.26 ± 3.14 mmol TE/g). The high antiradical activity of these extracts could be related to the high phenolic compound contents capable to scavenge radicals. Particularly, the high DPPH radical scavenging activity of the extract obtained from CHCI could be due to the anthocyanins. Similar to our findings, several studies have explained that the high antiradical activity of the extracts obtained from NADESs is consequence of their ability to perform high recovery of bioactive compounds (Bakirtzi et al., 2016; Oliveira et al., 2021).

3.3.2. Ferric reducing antioxidant power (FRAP)

The FRAP method is used to examine the ability of the antioxidants to exchange electrons in a purely redox reaction. The application of the FRAP assay aims to evaluate the capacity of phenolic compounds to reduce the ferric iron. The results of FRAP values of samples were shown in Table 3. The FRAP values of the samples varied significantly ($p < 0.05$) depending on the types of solvents. The extracts obtained from NADESs provided the ferric iron reduction activity ranged from 5.79 ± 0.57 to 83.08 ± 3.78 mmol ISE/g and nine of them showed better activity than distilled water (20.22 ± 1.02 mmol ISE/g), methanol (26.61 ± 2.01 mmol ISE/g) and ethanol (23.40 ± 1.14 mmol ISE/g). The high ferric iron reduction activity was determined with TARXYL, followed by ACSOR, CHAC, LASOR and CHLA. Although the efficiencies of these NADESs could be related to their ability to extract phenolic compounds from blackberry, NADESs providing high antiradical activity was found different from the solvents giving high ferric iron-reducing capacity. The same observation has been noticed when studying the antioxidant activity of various plants using NADESs as the extraction medium (Alsaud et al., 2021; Bakirtzi et al., 2016; Barbieri et al., 2020).

3.3.3. Iron chelating activity (FCA)

The metal chelation is important since metals like iron and copper are involved in the formation of reactive oxygen species (ROS) in vivo via Fenton and Haber-Weiss reactions such as superoxide radical (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) (Kehrer, 2000). Table 3 presented the iron-chelating capacities of the extracts obtained from NADESs and conventional solvents. The iron-chelating values of the samples varied significantly ($p < 0.05$) depending on the types of solvents. The lowest iron-chelating value was identified in the aqueous extract (0.04 ± 0.00 mg TE/100g) while the highest values were identified with ACSOR (4687.67 ± 83.58 mg TE/100g), CIXYL (1762.37 ± 83.58 mg TE/100g) and methanol (1595.21 ± 83.58 mg TE/100g). In addition to these, CHLA, LASOR, CHAC and MASOR exhibited also high iron-chelating capacity. Previously, Pavlović et al. (2016) have

Table 4
Anthocyanin compositions of blackberry obtained from NADESs and conventional solvents.

RT (min)	Compounds	NADESs													Conventional solvents		
		CHUR	CHGLU	CHXYL	CHLA	CHAC	CHCI	CHGLY	CHBUT	CHETHGLY	LASOR	ACSOR	TARXYL	TARSOR	CIXYL	MAXYL	MASOR	Distilled wat9er	Methanol	Ethanol
24.51	Cyanidin-3-glucoside	n.d.	855.40 ± 1.34f	773.77 ± 2.75i	875.55 ± 1.55e	832.21 ± 4.30g	887.81 ± 1.43d	852.58 ± 3.36f	802.21 ± 0.36h	837.95 ± 1.10g	876.04 ± 1.43e	965.56 ± 2.84b	688.78 ± 4.07k	1768.57 ± 4.34a	969.27 ± 5.20b	947.96 ± 5.89c	685.56 ± 5.24k	114.5 ± 0.87m	711.30 ± 8.43j	602.98 ± 7.40l
32.47	Cyanidin-3-rutinoside	n.d.	82.20 ± 1.52d	83.65 ± 1.11d	91.78 ± 1.88c	79.95 ± 2.44d	103.92 ± 0.93b	82.38 ± 1.90d	81.50 ± 2.97d	82.46 ± 1.81d	92.49 ± 0.20c	95.01 ± 1.05c	71.57 ± 0.21e	161.71 ± 0.18a	94.92 ± 0.89c	83.40 ± 2.87d	62.52 ± 1.21i	20.45 ± 0. 25g	67.84 ± 0.28f	58.09 ± 0.49h
35.97	Pelargonidin-3-glucoside	n.d.	13.93 ± 0.89bc	11.00 ± 1.02ef	9.20 ± 1.10 fg	5.65 ± 0.99h	2.06 ± 1.21i	11.01 ± 0.10de	4.85 ± 0.33h	7.72 ± 0.91g	8.80 ± 0.08 fg	13.62 ± 0.16bc	4.65 ± 0.36h	36.11 ± 1.56a	13.64 ± 2.18bc	13.83 ± 1.77bc	7.65 ± 0.30g	8.48 ± 0.16 fg	14.52 ± 0.24b	12.30 ± 0.08cd
39.77	Cyanidin chloride	n.d.	52.84 ± 0.15f	49.89 ± 0.39gh	79.98 ± 0.75b	47.19 ± 1.15ji	60.15 ± 0.90e	52.75 ± 0.24f	46.79 ± 0.50j	48.27 ± 1.33hij	63.45 ± 0.12d	62.12 ± 0.69d	49.03 ± 2.64hi	113.02 ± 0.14a	65.42 ± 1.01c	62.19 ± 0.62d	51.70 ± 0.14 fg	26.56 ± 0.23m	40.83 ± 0.51k	34.40 ± 0.10l

a-m = Different letters in the same row indicate significant differences between solvents ($P < 0.05$); n.d. = Not detected.

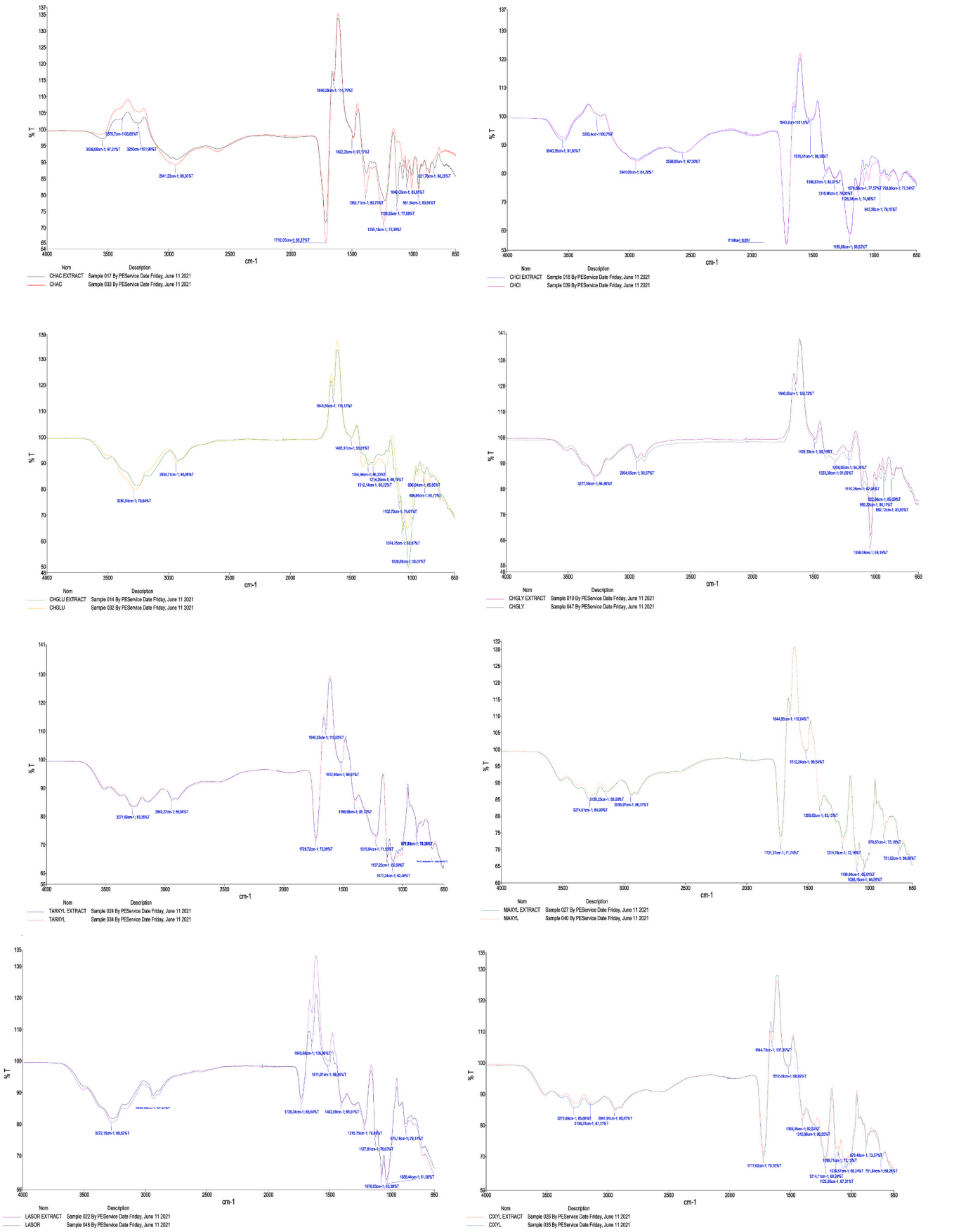

Fig. 5. FTIR spectra showing anthocyanin extraction reliability of CHAC, CHCI, CHGLU, CHGLY, TARXYL, MAXYL, LASOR and CIXYL.

determined the iron-chelating capacity varying between 1.55 and 8.86% from the leaves of different blackberry cultivars. Likewise, Junior et al. (2021) have reported the iron-chelating values varying between 17.12 and 261.12 mg/100g from the blackberry seeds. It can be observed that the extracts containing the organic acid-based NADESs presented higher iron-chelating activity, suggesting that the acid-based NADESs extracted particularly phenolic compounds having higher metal chelating capacity. Similar to our findings, the study of Oliveira et al. (2021) has proved that the organic acid-based NADESs had higher metal chelating capacity.

3.4. Characterization of anthocyanin profile

The profile of anthocyanins of blackberry was determined using the high-performance liquid chromatography (HPLC) coupled with diode-array detector (DAD) and the results were presented in Table 4. Four anthocyanins including cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin chloride and pelargonidin-3-glucoside were identified in all the solvents. Although the same anthocyanins were identified, their concentration varied significantly ($p < 0.05$) depending on the type of solvent. NADESs provided the highest recovery of anthocyanins when compared to water, methanol and ethanol. The organic acid-based NADESs mainly TARSOR, ACSOR, MAXYL, CIXYL, CHLA and CHAC provided the best recovery efficiency of anthocyanins from the blackberry samples. These results are in accordance with those mentioned above for the total anthocyanin content since these NADESs presented the lowest pH (0.47–1.32) which is appropriate for the extraction and preservation of anthocyanins. Similar to our findings, several studies have reported the best recovery of anthocyanin compounds from extracts obtained from the acidic NADESs (Bi et al., 2020; Fu et al., 2021; Zannou et al., 2020). In addition to acidic NADESs, CHGLY, CHBUT and CHETHGLY demonstrated also relatively high performance mainly due to their strong hydrogen-bonding network, polarity, and low viscosity. It is worth mentioning that no anthocyanin was determined with CHUR because of it high pH (9.99), since a pH higher than 7 is harmful to anthocyanins (Cvjetko Bubalo et al., 2016; Bosiljkov et al., 2017). Cyanidin-3-glucoside was the most dominant anthocyanin compound in blackberry for all the tested solvents, followed by cyanidin-3-rutinoside, cyanidin chloride and pelargonidin-3-glucoside, respectively. Our findings were found in agreement with the previous studies which have reported cyanidin-3-glucoside as the major anthocyanins of various blackberry cultivars (Da Fonseca Machado et al., 2018; Fan-Chiang & Wrolstad, 2006; Lee et al., 2015; Siriwoharn, Wrolstad, Finn, & Pereira, 2004).

The FTIR spectra of the most prominent NADESs in terms of the recovery of individual anthocyanins (TARXYL, CHAC, CHGLU, CHGLY, CHCI, MAXYL, LASOR and CIXYL) and their extracts were shown in Fig. 5. As can be seen, the extracts were the replicates of NADESs, indicating that NADESs inlaid the solutes as their own components. Representative spectra located at 1720-600 cm^{-1} region could be associated to C–O stretching of the aromatic ring, deformation of the two phenyl rings in the anthocyanins (Navikaite, Simanaviciute, Klimaviciute, Jakstas, & Ivanauskas, 2016), –C═C- stretching vibrations in the phenyl ring (Romera-Fernández et al., 2012), and –C═C- in the aromatic ring of benzopyran which is the skeleton molecule of anthocyanins (Klimaviciute, Navikaite, Jakstas, & Ivanauskas, 2015). The anthocyanin cyanidins could be specifically assigned to the peaks at 3400-3100 cm^{-1} (OH), 2900-2840 cm^{-1} (C–H aliphatic), 1660 cm^{-1} (C═C aromatic) and 870-675 cm^{-1} (CH aromatic) (Wahyuningsih, Wulandari, Wartono, Munawaroh, & Ramelan, 2017).

4. Conclusion

An efficient greener method was investigated for the extraction of blackberry anthocyanins and antioxidant activity using sixteen NADESs constituted of choline chloride-urea, choline chloride-sugars, choline chloride-organic acids, choline chloride-polyalcohols and organic acids-sugars were synthesized. Water, methanol and ethanol were used as conventional solvents for comparison. The highest total phenolic content (9.35 ± 0.39 mg GAE/g), total flavonoid content (104.72 ± 5.17 mg ECE/100g), total anthocyanin content (115.37 ± 0.43), antiradical activity (68.77 ± 2.29 mmol TE/g), reducing power (83.08 ± 3.78) and metal chelating activity (4771.25 ± 83.58 mg TE/100g) were obtained with NADESs. NADESs such as CHGLU, CHGLY, CHAC, CHLA, CHBUT and CHETHGLY displayed the highest performances. Particularly, the more acidic NADESs such as TARXYL, CIXYL, MAXYL, CHCI, LASOR and CHAC provided the highest total anthocyanin content and extracted higher quantities of cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin chloride and pelargonidin-3-glucoside. This study revealed that NADESs are a green and efficient alternative for obtaining biocompatible extracts that are selectively enriched in bioactive compounds.

Declaration of conflict interests

Authors have no conflict of interests.

CRediT authorship contribution statement

Oscar Zannou: Conceptualization, Methodology, Investigation, Software, Data curation, Writing – original draft. **Ilkay Koca:** Data curation, Visualization, Supervision, Software, Validation, Writing – review & editing.

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