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Green Separation of Bioactive Natural Products Using Liquefied Mixture of Solids

Siti Zullaikah, Orchidea Rachmaniah,
Adi Tjipto Utomo, Helda Niawanti and Yi Hsu Ju

Additional information is available at the end of the chapter

Abstract

Bioactive natural products are secondary metabolites of plants and animals generated through various biological pathways. They are the main sources of new drugs, functional food and food additives. Since their contents in plant and animal tissues are extremely small compared to those of primary metabolites, the separations of bioactive principles from complex matrixes are often the inherent bottleneck in the utilization of bioactive natural products. A novel separation technique based on a liquefied mixture of solids at its eutectic compositions is presented in this chapter. The mixture can be prepared from natural primary metabolites and therefore can be considered as a green solvent. The separation of bioactive compounds (γ-oryzanol) from rice bran oil-based biodiesel using green methods with minimum energy requirement is discussed. Other applications for separations of alkaloid and phenolic compounds from their plant matrices are also presented. Different raw materials require different separation techniques due to the presence of different impurities, and the current trend is to use green methods with minimum energy requirement. This overview of recent technological advances, discussion of pertinent problems and prospect of current methodologies in the separation of bioactive natural products may provide a driving force for the development of novel separation techniques.

Keywords: green separation, bioactive natural products, deep eutectic solvent, natural deep eutectic solvent

1. Introduction

Bioactive compounds of plants, also known as natural products, are produced as secondary metabolites. Unlike primary metabolites, such as carbohydrates, proteins, fats, amino acids,

nucleic acids and organic acids, which are essential to perform the metabolic rules involved in the life process, they have no apparent direct functions in growth, development and reproduction. They are often differentially distributed among limited groups of plants and only present in very low quantities in plants. Though, in principle, they are inessential to life, many secondary metabolites found in plants have roles in defense against predators (herbivores, pests and pathogens), competition and facilitating the reproduction process. However, many of them still remain unknown in their functions. Previously, secondary metabolites were generally thought to be waste products of plants without apparent function. Nowadays, they represent an important source of biological active compounds which are very important for the development of food and pharmaceutical industries.

Since their content is small and different raw materials require different isolation techniques due to the presence of different impurities, the extractions of bioactive principles from complex matrixes are often the inherent bottleneck in the utilization of bioactive natural products. The extraction techniques can be classified into conventional and modern ones [1]. The conventional techniques include maceration, percolation, Soxhlet extraction and solvent extraction. They are typically characterized by long extraction time, high cost due to the requirement of large volume of solvents, low yield and the use of toxic and flammable solvents. The modern techniques include enzyme-assisted extraction, ultrasound-assisted extraction, microwave-assisted extraction, subcritical and supercritical fluid extraction and high pressure-assisted extraction. They generally have shorter extraction time, lower cost, higher purity of the extracted compounds and much better efficiency [2]. However, there are still issues associated with conventional and modern extraction techniques, including the toxicity of solvent, thermal instability, solubility and poor selectivity. In addition, the type and concentration of solvents, their moisture contents, recovery of bioactive compounds and changes of bioactive compounds during extraction due to ionization, hydrolysis, esterification and oxidation need to be considered [3]. Water can be used as solvent in the extraction of bioactive compounds. However, water is only effective to extract polar and hydrophilic bioactive compounds but less effective to extract non-polar and hydrophobic ones. In addition, impurities extracted by water become another problem for further purification steps.

Green solvents have been explored to replace traditional hazardous solvents that are used extensively in industry. To be qualified as a green solvent, a solvent should be non-toxic, non-volatile, biodegradable without generating any toxic and persistent metabolite, inflammable, recyclable, relatively cheap and available in a large quantity [4–6]. Water at sub- and supercritical conditions and CO_2 at supercritical condition are examples of green solvents that have been used in industrial scale.

Recently, ionic liquids (ILs) have been developed as green solvents. Ionic liquids are molten salts, mixtures of bulky and asymmetric organic cations and organic or inorganic anions, with melting points usually below 100°C [2, 7]. They have some attractive attributes, such as non-flammable, high thermal and chemical stabilities and low vapor pressure [1, 4, 8–10]. The combination of different cations and anions makes ILs have a tunable nature, i.e. polarity and other properties

with their own unique structures and properties. Therefore, there has been an increase in the use of ILs as green solvent for the extraction, separation and purification of natural bioactive compounds [2]. However, there are concerns about the application of ILs related to the toxicity of these compounds, their potential effects on health and the environment and the high cost associated with their synthesis and purification requirements [8, 11].

To overcome the drawbacks of ILs, deep eutectic solvents (DESs) have been developed. They have physicochemical properties similar to those of ILs. In addition, DESs are biodegradable, less toxic and cheaper than ILs [12]. DESs are formed from mixtures of two or more Lewis acids and bases or Bronsted-Lowry acids and bases that have the lowest freezing points compare to their starting constituents [13]. The physical structures of some DESs are similar to those of ILs. However, DESs in general are different in terms of the source of the starting ingredients and the chemical formation process.

2. Eutectic solvents

Deep eutectic solvent (DES) is a eutectic mixture of two or more compounds which has a melting point much lower than either of the individual components [6, 14, 15]. A eutectic mixture is the condition when the molar ratio of the component gives the lowest melting point as represented in **Figure 1**. DES was first introduced by Abbott et al. [14] who studied the properties of choline chloride (ChCl)/urea mixture. Both ChCl and urea have melting points of 302 and 133°C, respectively. However, at the eutectic composition (1:2 ChCl/urea molar ratio), the mixture melts at 12°C making it liquid at room temperature.

Typically, DESs are mixtures of quaternary ammonium halide salts and hydrogen bond donors (HBDs). Various quaternary ammonium halide salts and HBDs which can form DESs are shown in **Figure 2**. One of the most widely used ammonium quaternary salt for DESs
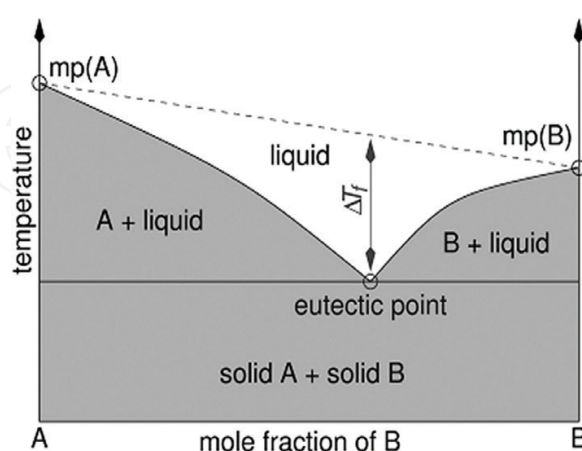

Figure 1. Schematic representation of a eutectic point on a two-component phase diagram [15].
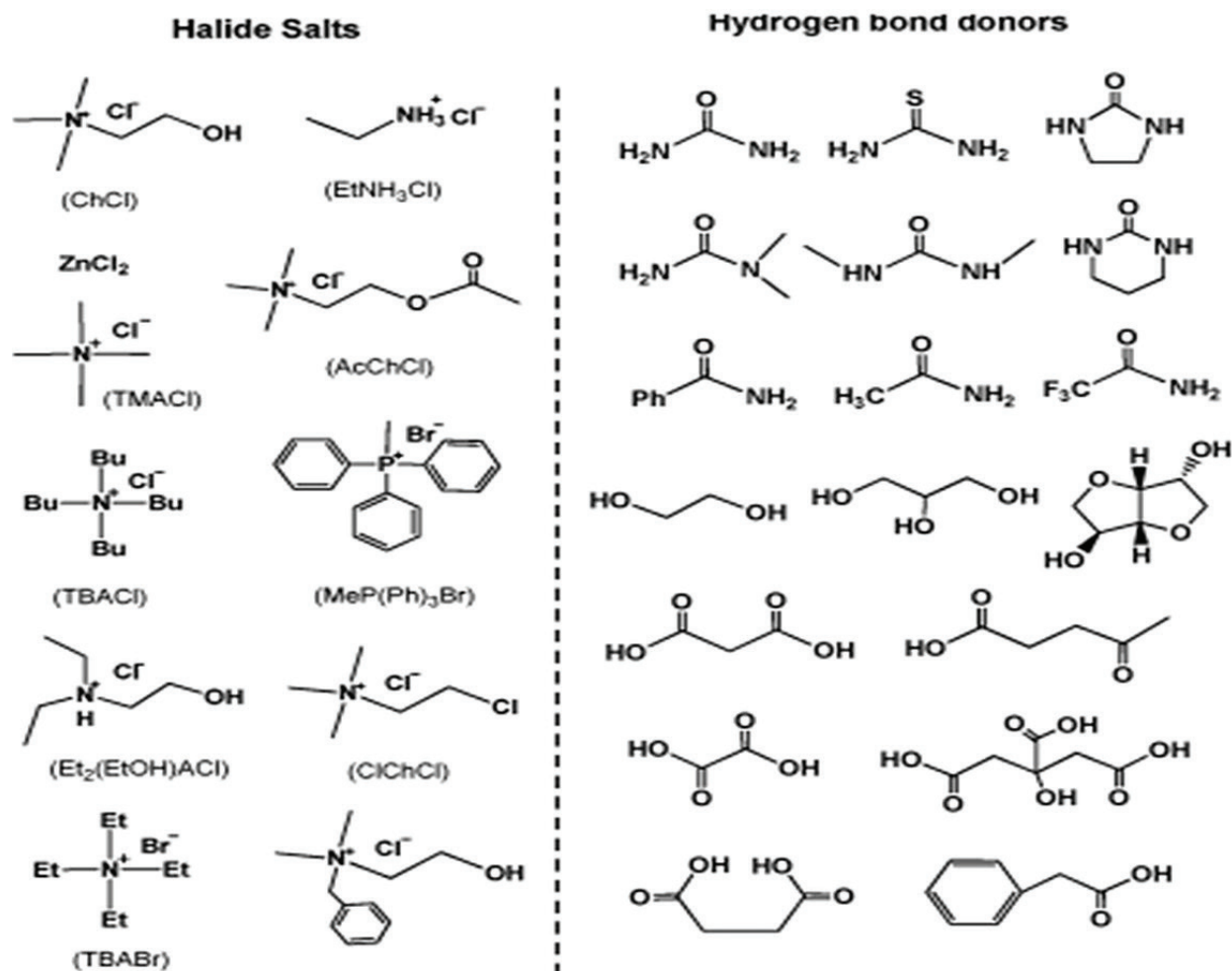
Figure 2. Halide salts and HBD for DES [13].

formation is ChCl. ChCl is cheap, biodegradable, non-toxic and can be easily extracted from biomass or synthesized from fossil fuel, while the HBD could be amides (e.g. urea) [14], carboxylic acids (e.g. oxalic acid), alcohols (e.g. glycerol) [13, 16], sugars or sugar analogues, amino or organic acids and alkylsulfates or alkyl phosphates.

The main advantage of DESs over the previous generation of ILs is that they are easier and simpler to make. The solids are mixed in gentle heat until they melt, and when they cool down, they remain in the liquid form. No purification steps are required since there is no formation of new salt and the final purity is determined by the purities of the starting materials. The design of DESs is simpler and more flexible since no reaction takes place, and therefore it does not have any strict stoichiometry limitation. The interaction between HBD and hydrogen bond acceptor (HBA) will form a liquid in their relative molar composition. The behaviors and properties of DESs can be tuned by varying the HBD and its molar ratio in the mixture [13]. DESs have moderate polarity, stability and distributed negative charge like ILs, but they are biodegradable, readily available and less toxic since DESs can be based on bulk natural product such as carbohydrates (fructose, glucose, mannose, maltose and α-cyclodextrin), sugar alcohols (sorbitol) or citric acids with urea (or N,N-dimethylurea) and inorganic salts (NH_4Cl and $CaCl_2$) [10, 17].

Another term of DESs was introduced by Gutiérrez et al. [16] as low transition temperature mixtures (LTTMs). They are the right combinations of different molar ratios between HBD and HBA, such as lactic acid/alanine = 9:1, lactic acid/ChCl = 2:1, lactic acid/histidine = 9:1, etc. The formed liquid mixtures have glass transitions instead of melting points [18]. A complete characterization of physical properties (density, viscosity, surface tension, glass transition temperature) of LTTMs, i.e. lactic acid/ChCl = 2:1, was reported by Francisco et al. [18].

Recently, Choi et al. [19] have first reported a large number of DESs which were mixtures of ChCl with any primary metabolites, e.g. sugars, sugar alcohols, organic acids and amino acids. This type of DESs is termed as natural deep eutectic solvents (NADESs). NADESs derived from major compounds always present in all microbial, mammalian and plant cells form liquid crystals. Therefore, NADESs are believed as the third liquid phase present in the cells, in addition to the already considered known phases, i.e. water and lipid [membranes] are responsible for transporting of numerous compounds with intermediate polarity in high concentration that neither dissolve in the lipid nor in the aqueous phase. Rutin, a flavonoid which is barely soluble in water, has a solubility of 0.28 mmole/mL in glucose-choline chloride-water (GCH, molar ratio = 2:5:5), thus 50–100 times higher than that in water [20], whereas, paclitaxel and ginkgolide B, which are completely water-insoluble compounds, have solubilities of 0.81 and 5.85 mg/mL, respectively, with the same NADES type [19]. In the case of macro molecules, such as DNA and starch, they also show higher solubilities in NADESs than those in water, i.e. 1.20 and 7.55 g/mL, respectively. These facts are in line with the hypothesis of the existing alternative liquid phase to water in nature of poorly water-soluble molecules including high molecular weight molecules. Though GCH (2:5:5) has polarity close to water, GCH as a NADES shows different performance than water. It indicates that NADESs have huge potential for many practical applications since they can be designed as tailor-made solvents.

3. Extraction of bioactive compounds from rice bran oil (RBO)-based biodiesel by choline chloride-based deep eutectic solvent

Rice bran is a promising raw material for biodiesel production. It is relatively cheap, abundant and traditionally used as cattle food. The annual worldwide production of rice bran oil (RBO) could reach 8 million tons if all rice bran produced is harnessed for oil production [21]. RBO is rich in naturally occurring biologically active and antioxidant compounds, such as phytosterols, γ-oryzanol, tocopherols and tocotrienols (tocols) [22]; however, the refinery of crude RBO requires extra processing steps due to high concentrations of free fatty acids (FFA), unsaponifiable matter and dark color [21] making it uncompetitive against other edible cooking oils, such as palm oil, soybean oil and rapeseed oil.

Since RBO contains high FFA, conventional process to produce biodiesel using base catalyst is unsuitable to convert RBO into biodiesel (fatty acid methyl esters, FAME) due to the formation of soap. Several methods have been proposed to convert crude RBO into FAME, i.e. a two-step acid-catalyzed process [23], a three-step method using both acid and base catalysts [24] and a supercritical methanol method [25]. More recently, in situ process to produce biodiesel from rice bran without any pretreatment has also been proposed by Zullaikah et al. [26].

Crude biodiesel produced from RBO using acid-catalyzed methanolysis method typically contains about 89% FAME, 4% triglycerides (TG), 4% diglycerides (DG), 0.3% monoglycerides (MG), 0.05% FFA and 2.55% bioactive compounds, mainly phytosterols and γ-oryzanol. To meet biodiesel standard as fuel (such as [27]), purification process is required to increase FAME content to at least 96.5% and decrease unreacted oil (TG, DG, MG and FFA) contents. Crude biodiesel produced from RBO through acid-catalyzed methanolysis method has different impurity compositions to those produced from edible oil (palm oil, soybean oil and rapeseed oil) using base catalyst, and therefore a different purification process is required. Besides that, since crude biodiesel from RBO contains bioactive compounds, a purification process which is able to capture those bioactive compounds will be of interest. According to Ju and Zullaikah [22], bioactive compounds were not much degraded during acid-catalyzed methanolysis. These bioactive compounds could subsequently be isolated and sold separately as high-value by-products and therefore could reduce the production cost of biodiesel.

DES is a promising solvent to be employed in the purification of crude biodiesel since it is inflammable, non-toxic, biodegradable and considered as a green solvent [12]. One of the most commonly used DESs for biodiesel purification process is a mixture of choline chloride (an ammonium quaternary salt) and ethylene glycol as HBD at a molar ratio of 1:2. The mixture of ethylene glycol and ChCl is called ethaline, and some physical properties of ethaline were shown in **Table 1**. Ethaline has two interactions. The first is between the chlorine anion and the hydroxyl hydrogen atom of choline, and the second is between the anion and the hydroxyl hydrogen atoms in ethylene glycol [28]. Ethaline has a strong interaction with unreacted oil (DG, MG and FFA) and unsaponifiable matter (bioactive compounds) from crude RBO-based biodiesel due to the presence of hydroxyl groups on those compounds. On the other hand, the interaction between ethaline and FAME is relatively weak since FAME has no hydroxyl group.

Based on the previous research [29], ethylene glycol interacted into each other by making hydrogen bonding in cyclical pattern and the distance of H-O bond was 1.944 Å, whereas, ChCl in crystalline structure has three bonds consisted of C-N, C-O and C-C, and the distance of each bond is 0.01 Å. ChCl is difficult to convert into liquid at room temperature due to the small distance of ChCl bonds. Based on Wagle et al. [29], DES from ChCl and ethylene glycol has two interactions of C-H-O. The first one was between oxygen from ethylene glycol and methyl proton from ChCl, and the distance was 2.146–2.440Å. The

Melting point (K)[a]	207.15
Viscosity (cP)[b]	36
Conductivity (mS/cm)[b]	7.61
Density (g/cm)[b]	1.12
Surface tension (mN/m)[b]	49

[a][30].
[b][15].

Table 1. Physical properties of ethaline (molar ratio of ChCl/ethylene glycol = 1:2).

second one was between hydrogen in ethylene glycol and Cl^- in ChCl, and the distance was 2.271–2.474 Å. Cl^- as anion in ChCl forms a centerpiece by interacting with five hydroxyl groups, one hydroxyl group of choline cation and four hydroxyl groups from two ethylene glycol molecules.

As a novel green solvent, a combination of mechanisms and molecular structure of DES is still unknown [31]. Therefore, FT-IR analysis was conducted in this study to determine the functional groups of DES, and the results are shown in **Figure 3** and **Table 2**. The peak in the region of 3200–3500 showed the presence of O-H groups in choline chloride and ethylene glycol-based DES which is in agreement with that reported by Aissaoui [32]. **Figure 3** describes that there is a shift of the O-H stretching band in the DES compared to that in the choline chloride and ethylene glycol. This shift is due to the electrons of oxygen that are transferred to the hydrogen bonds making the constant force lower and resulting in a change in the vibrational state. The shifting of O-H stretching vibration indicates the presence of hydrogen bonds in DES [32]. The peaks in the region of 3000-2800 on choline chloride, ethylene glycol and DES show the presence of C-H, CH_3 and CH_2 stretching bands. Meanwhile, the N-H stretching bands overlap with the C-H vibrational bands in the region of 3000-2800 cm^{-1} [33]. As shown
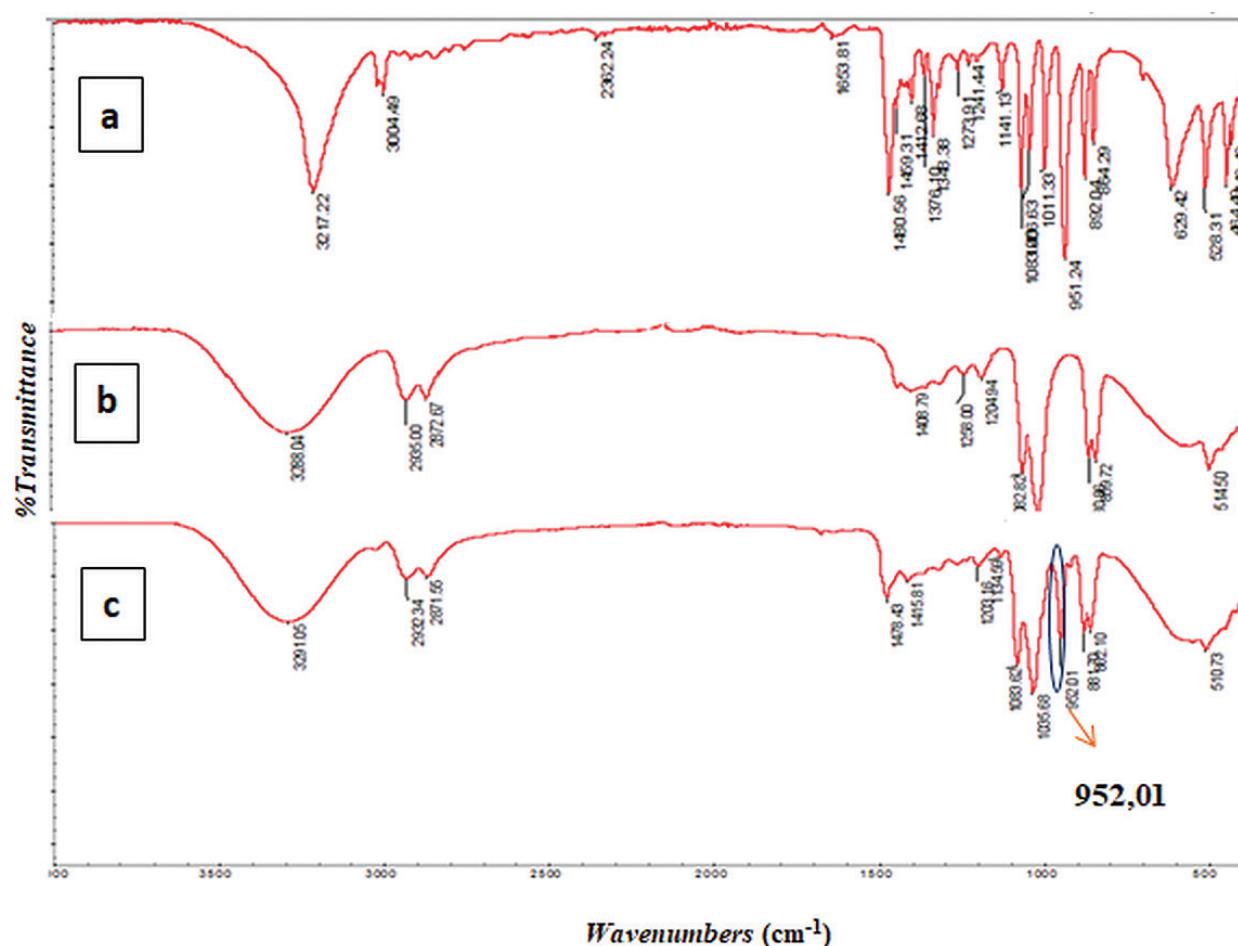

Figure 3. FT-IR analysis: (a) choline chloride, (b) ethylene glycol, (c) ethaline (molar ratio of choline chloride/ethylene glycol = 1:2).

Wavenumber	Functional group
3200–3500	O-H (alcohol)
2845–3000	C-H *stretching*, CH$_2$ *stretching*, CH$_3$ *stretching*
496–700	C-X stretching (X = F, Br, Cl or I)
1210–1150	Tertiary amine (C-N)
1000–1350	C-C *stretch*
[a][33].	

Table 2. Wavenumber and functional group of ethaline analysis by FT-IR[a].

in **Figure 3**, the stretching vibration at 2500-3100 regions in choline chloride is invisible after the formation of DES. The presence of Cl$^-$ in DES is shown at 600 and 408 cm^{-1}. **Figure 3** also describes that the DES had a vibration pattern similar to ethylene glycol as HBD, except that the peak is at 952.01 cm^{-1}. The peak appears on DES is in the region of 935-955 cm^{-1} indicating the identity of ammonium structure of DES [32]. The FT-IR analysis describes that the establishment of DES does not lead to the formation of new functional groups in the mixture.

The purification process of crude RBO by using DES is relatively simple and can be described as follows. Crude RBO-based biodiesel and DES were mixed in a stopper glass (50 mL) at a certain molar ratio (1:8). The mixture of biodiesel and DES was heated at a certain temperature (30°C) under stirring at 300 rpm. Afterwards, the mixture was let to settle for 2 h at ambient temperature so that two layers were formed. The upper layer was biodiesel (FAME)-rich phase and the bottom layer was DES-rich phase containing biodiesel impurities including bioactive compounds. The biodiesel-rich phase (upper layer) was then separated from DES-rich phase by using a separator funnel. There are several factors that influence the purification process, such as extraction time, extraction temperature and molar ratio of DES/RBO-based biodiesel. However, this chapter only discusses the effect of extraction time on FAME recovery, removal of unreacted oil and bioactive compounds and the final biodiesel composition.

Figure 4 shows the effects of extraction time on the biodiesel recovery and the removal of unreacted oil and bioactive compounds. Extraction time is one parameter that influences liquid–liquid extraction. The unreacted oil and bioactive compounds diffuse from biodiesel-rich phase to DES-rich phase. More unreacted oil and bioactive compounds migrate to DES-rich phase with longer contact time between crude biodiesel and DES. FAME recovery {(FAME in product/FAME in sample)×100%} also increased from 63.38 to 87.31% as the extraction time was extended from 15 to 240 min as shown in **Figure 4**.

The removal efficiency of unreacted oil and bioactive compounds has similar trend with time as shown in **Figure 4**. However, the removal efficiency of each compound is different. Since TG have no OH$^-$ group, their removal efficiency was lower than the other compounds with OH$^-$ groups, such as DG, MG, FFA and bioactive compounds. TG removal efficiency was practically unaffected by extraction time. TG removal at extraction times of 15 and 240 min were 41.32 and 39.69%, respectively. However, the removal efficiencies of unreacted oil (DG, MG and FFA) and bioactive compounds increased with time. DG removal increased from
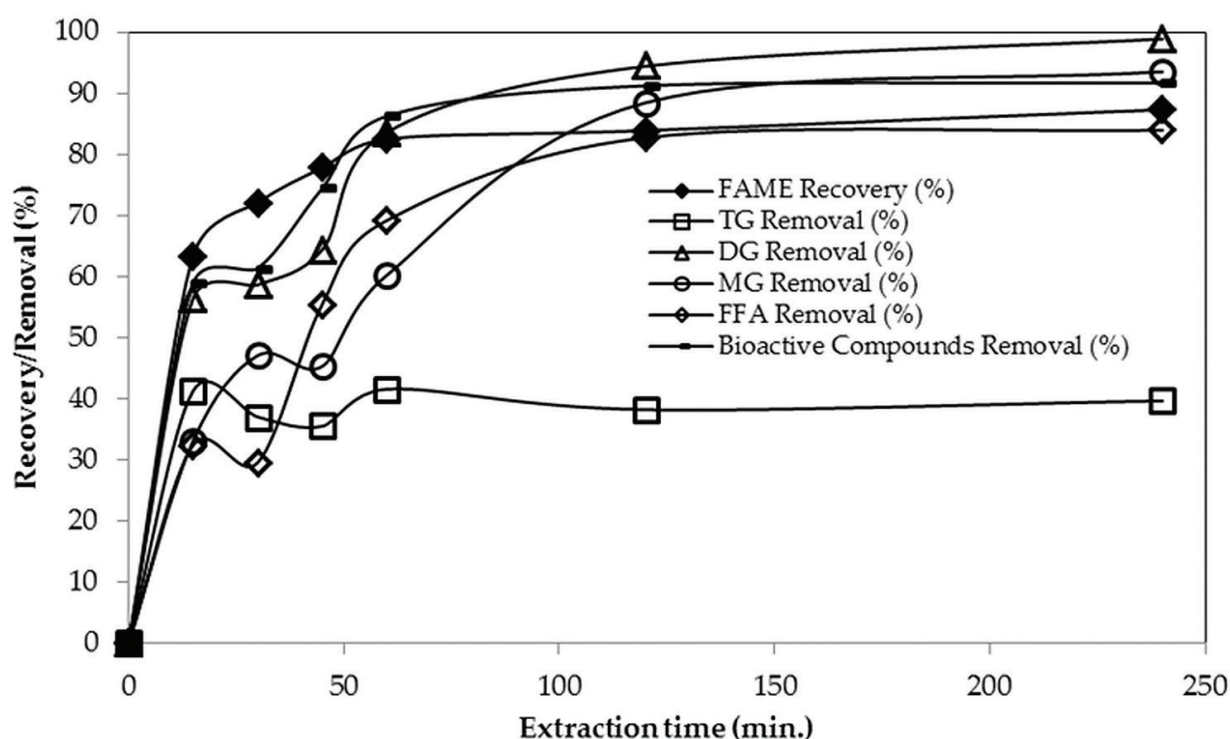
Figure 4. Effect of extraction time on recovery of FAME, unreacted oil and bioactive compound removal. Operation conditions, T = 30°C; molar ratio of ChCl, ethylene glycol = 1:2; and molar ratio of RBO-based biodiesel, DES = 1:8.

56.42 to 98.88% as the extraction time was increased from 15 to 240 min, while that of MG increased from 33.15 to 93.52%. The removal efficiency of FFA was lower than DG and MG, even though they have OH- group. This is probably because FFA content in RBO-based bio-diesel was much lower than those of DG and MG. Since bioactive compounds in RBO, such as γ-oryzanol, phytosterols and tocols, have OH- group, they can make hydrogen bonding with DES, and their removal efficiencies were high. The removal of bioactive compounds increased from 58.91 to 91.70% with the increasing extraction time from 15 to 240 min.

The effect of extraction time on the contents of FAME, unreacted oil and bioactive compounds is shown in **Figures 5** and **6**, respectively. FAME content increased with extraction time (**Figure 5**), while those of unreacted oil and bioactive compound content decreased with extraction time (**Figure 6**). Longer extraction time provides longer contact time between DES- and RBO-based biodiesel. Therefore, more unreacted oil and bioactive compounds diffuse to DES through the formation of hydrogen bond. Since unreacted oil and bioactive compounds were removed from RBO-based biodiesel, the FAME content increased. The FAME content after 240 min purification using DES was higher than 96.5%, exceeding that specified by the European biodiesel standard [27]. FFA is undesirable in biodiesel since it causes negative impacts, such as less oxidation stability and corrosion of vital engine components. The FFA content in biodiesel is characterized by acid value. The acid value of RBO-based biodiesel produced using acid-catalyzed methanolysis method was 0.098 mg KOH/g. This acid value was already lower than the maximal acid value stated in several biodiesel standards such as EN 14214 [27] (0.5 mg KOH/g). FFA content in biodiesel after purification was decreased from 0.05 to 0.01% (**Figure 6**). This showed that the removal efficiency of FFA using DES as
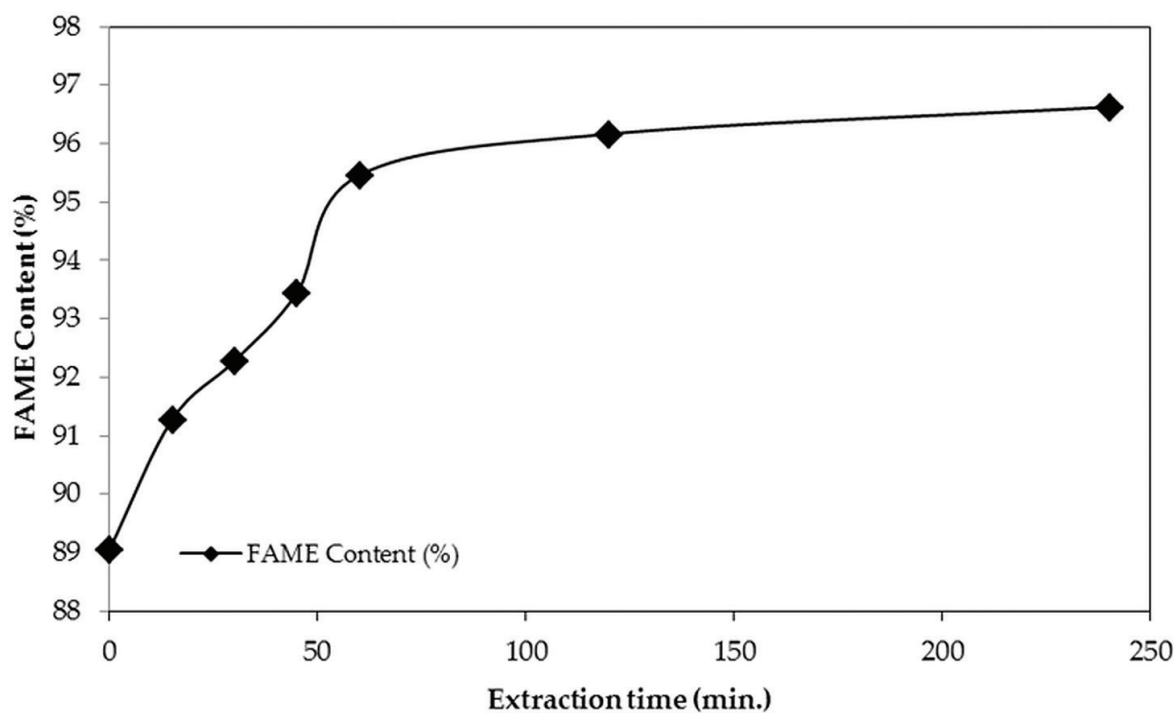
Figure 5. Effect of extraction time on FAME content. Operation conditions, T = 30°C; molar ratio of ChCl, ethylene glycol = 1:2; and molar ratio of RBO-based biodiesel, DES = 1:8.

Figure 6. Effect of extraction time on unreacted oil and bioactive compound content. Operation conditions, T = 30°C; molar ratio of ChCl, ethylene glycol = 1:2; and molar ratio of RBO-based biodiesel, DES = 1:8.

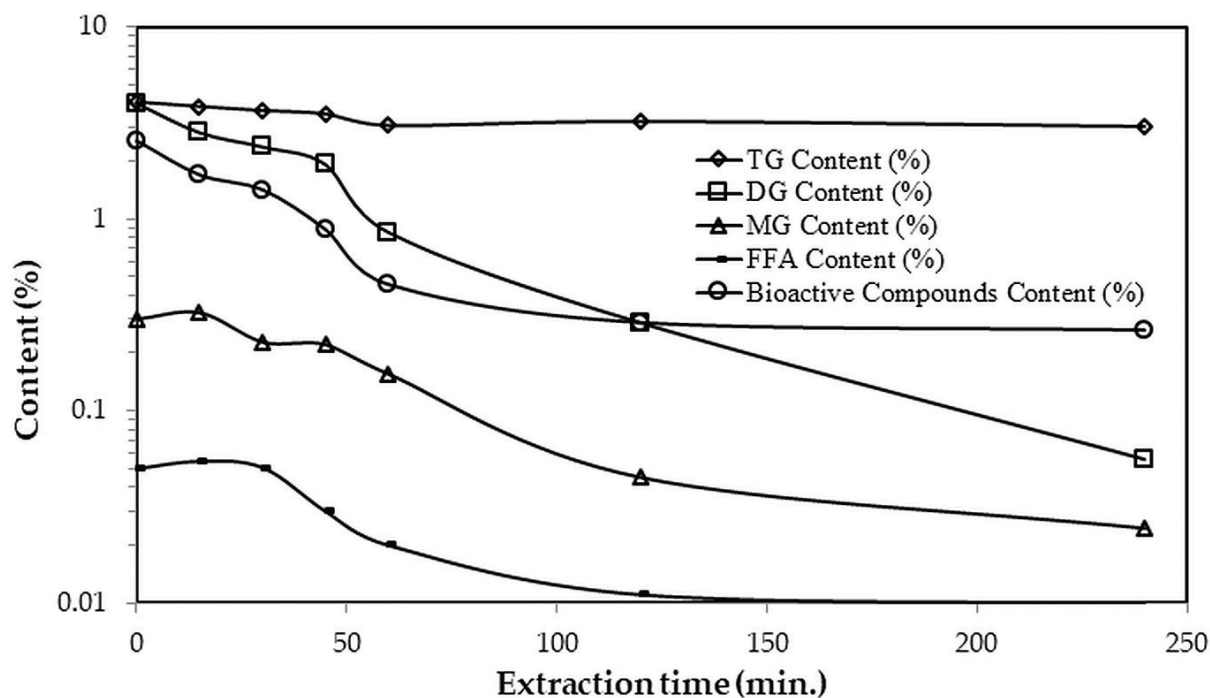| Property	Unit	Experimental value	EN 14214 [27]
Ester content	% (m/m)	96.65	Min 96.5
Acid value	mg KOH/g	0.02	Max 0.5
MG content	% (m/m)	0.02	Max 0.8
DG content	% (m/m)	0.06	Max 0.2
TG content	% (m/m)	3.02	Max 0.2

[a]Operation condition, T = 30°C; molar ratio of ChCl, ethylene glycol = 1:2; molar ratio of RBO-based biodiesel, DES = 1:8, 240 min extraction time.

Table 3. Composition of biodiesel after purification using DES[a].

extraction solvent was high. This case shows that the extraction time can affect the extraction process of FFA in biodiesel.

The composition of biodiesel after purification using DES is compared to that specified by biodiesel standard, EN 1424 [27], as shown in **Table 3**. The ester content, acid value, MG content and DG content meet the values specified by EN 14214 [27]. However, TG content was much higher than those specified by EN 14214 [27]. This is because TG does not have OH^{–} group so it does not interact with DES used in this experiment. This can be overcome by using a multiple-step separation technique.

According Niawanti and Zullaikah [34], γ-oryzanol in the upper layer (biodiesel-rich phase) was decreased with extraction time from about 4% initially to about 1% after 240 min of extraction. The lowest γ-oryzanol content of 1.18% was obtained after 240 min of extraction time. The reason of this phenomenon is that more γ-oryzanol move from RBO-based biodiesel to DES with longer extraction time. The molar ratio of biodiesel to DES also influences the removal efficiency of γ-oryzanol. The higher molar ratio of DES to RBO-based biodiesel leads to higher removal efficiency of γ-oryzanol since more γ-oryzanol molecules are bound to DES molecules through hydrogen bonding.

4. Extraction of phenolic and alkaloid compounds using natural deep eutectic solvent

In recent years, many herbs and natural compounds have increasingly been receiving public interest as complementary and alternative medicines. The natural product curcumin 1,7-bis(4-hydroxy-3-methoxy phenyl)-1,6-heptadione-3,5-dione is a dietary phytochemical obtained from the dried rhizomes of the turmeric plants. It is a natural bioactive compound that has demonstrated both antioxidant and therapeutic anticancer capabilities. However, it is not yet fully used clinically due to its inherent limitations, i.e. sparing solubility in water and low bioavailability. Curcumin (C) is extracted from *Curcuma Sp.*, i.e. *Curcuma longa*,

Curcuma zedoaria and *Curcuma manga,* a plant of the ginger family, with other two curcuminoid compounds: demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC).

Traditionally curcumin has been used as food coloring, flavoring and preservative. Because of its wide spectrum of biological activity, an extensive number of studies have been focused on curcumin. Recently, curcumin has also been shown to display antioxidant, anticancer, antiviral, anti-infectious and anti-amyloidogenic properties. Numerous methods to isolate curcumin as well as other curcuminoids from *C. longa* rhizomes have been reported, such as conventional solvent extraction, hot and cold percolation, the use of alkaline solution and insoluble salt, supercritical carbon dioxide extraction, microwave-assisted extraction and ultrasonic-assisted extraction techniques. However, only a few of them use either green solvent or green process such as pressurized hot water extraction [35]. In the contrary, volatile organic solvents, e.g. methanol, ethanol, acetone and hexane, are still widely applied.

The capabilities of NADES to extract and stabilize bioactive compounds have been investigated by several authors. The solubilities of secondary metabolites such as rutin, quercetin, cinnamic acid, carthamin, taxol and ginkgolide B in different types of NADES have been studied by Choi et al. [19] and Dai et al. [20], while the stabilization ability of NADES on unstable natural colorants, carthamin and anthocyanins, during heating, storage and exposure to light has been reported by Dai et al. [36]. In addition, Bajkacz and Adamek et al. [37] also showed that NADES can be applied for isoflavone extraction. NADES can also be applied for protein stabilization [38], bioavailability improvement [39], antimicrobial agent [40] and bioactivity enhancement of plant extract [41]. Most of them used choline chloride-based NADES, the best combination of NADES which is suitable with their studied compounds. The broad range utilization of NADES showed that NADES leads a novel application in food and pharmaceutical industry.

The selection of solvent for extraction, i.e. liquid-liquid extraction (LLE), depends on its physical properties such viscosity, density and miscibility. It is convenient to select solvent with low viscosity to facilitate mixing as well as maximizing solvent penetration to the plant matrix but with a large density difference for the separation process. The inherent viscous properties of NADESs differ enormously according to their composition, but in all cases, it can be reduced by the addition of a certain amount of water. It should be noted that the addition of water changes the properties of NADESs, i.e. polarity, density and solubilizing and stabilizing capability. However, excessive dilution of NADESs, ca. approximately >50% weight of water, disrupts the special structure of NADESs due to the loss of the existing hydrogen bonds [20]. The viscosities of NADESs can also be decreased by increasing temperature. Generally, NADESs composed of sugar are the most viscous, while choline chloride-based NADESs are less viscous, while the glycerol-based NADESs are the least. Common efforts to minimize the resistance of viscosity and improving the extraction rate, such as mechanical agitation, microwave and ultrasound-assisted extraction, can be used with NADES.

To figure out the broad application of NADES in natural product extraction, this chapter documents the application of NADES on the extraction of curcumin, a low solubility phenolic compound in water, and galantamine, an alkaloid of acetylcholine inhibitor.

Curcuma zedoaria that contains 1.96% ± 0.07% of dry weight of curcuminoids was kindly donated by Sari Herbal (Sukun, Malang, Indonesia). It was chosen in this study since it is traditionally believed as an anticancer medicine in Indonesia. Twelve NADESs were used as solvents for extracting curcuminoids from fine powder of *C. zedoaria*, representing different types of NADESs: (1) ionic type, consisted of organic acids, i.e. citric acid, malic acid and lactic acid, and basic compounds such choline chloride or betaine; (2) neutral type, no ionic constituent, mixed of polyalcohol, i.e. glycerol, glycine, 1–2-propanediol and sugars; (3) acidic type, consisted of neutral compounds such sugars and acidic compounds; (4) basics type, consisted of basic compounds; and the last is (5) amphoteric type, consisted of combination of amino acids and sugars, polyalcohol or acidic compounds. In the case of ionic type of NADESs, it is represented by CCCA-H_2O and CCMA-H_2O; FS-H_2O and FG-H_2O are neutral type and CAS-H_2O and MAS-H_2O for the acidic type, whereas CCGo-H_2O, CCG-H_2O and CCF-H_2O are the basic type. Meanwhile, the amphoteric types were excluded in this study.

A simple extraction protocol was developed to test the capability of NADES to solubilize curcuminoids from plant matrix. The powder of *C. zedoaria* was mixed with NADES in a bottle with cap (powder/NADES ratio = 20 mg powder/3 mL NADES) and stirred (350 rpm) at 40°C for 24 h. Triplicate samples of the resulting solution were diluted with water and analyzed with HPLC-DAD at a wavelength of 421.4 nm. The NADESs were prepared according to Dai et al. [20] with slight modification, i.e. by using freeze-drying instead of vacuum evaporation. The liquefied solid mixture can be called as NADES, when after the freeze-drying process it remains liquid and is visually clear and transparent with no precipitation and crystallization that are formed. It can be kept until a year without any changes in appearances and physical properties, i.e. density and viscosity. In addition, the purity of the individual component of NADES does not affect the NADES properties. As shown in **Figure 7**, the FT-IR spectra of CAS-H_2O prepared with different grades (purity) of citric acid are similar indicating that there are no structural changes.

In analogy with DES, the two components of NADES are particularly bonded by hydrogen bond [19]. The cross-correlation between sucrose and malic acid was observed by ^1H-^1H-nuclear Overhauser enhancement spectroscopy (NOESY). It revealed molecular interactions of protons on the C2 and C3 positions of malic acid with those on the C1 and C2′ of sucrose [19]. This analysis also suggests that water might also participate in the formation of supermolecular structure of NADES [19, 20].

In the case of acidic type of NADESs, e.g. CAS-H_2O and MAS-H_2O, a yellowish color will be observed right after the extraction process takes place. It will be deepened along the elapsed extraction time due to the caramelization of sucrose promoted by the presence of acid. The pH of both CAS-H_2O and MAS-H_2O is two (measured at 10x dilution with Aquadest). The caramelization reaction is the hydrolysis of sucrose by protonation of the glycosidic linkage [42]. Though the extraction process was only heated at 40°C, the effect of acidic condition is comparable with the effect of heating at high temperature at neutral pH [43]. However, neither the density nor viscosity of CAS-H_2O and MAS-H_2O was significantly affected by the hydrolysis reaction (**Figure 8**). Nevertheless, sugar hydrolysis was not observed in other NADESs that consist of sucrose such as in the neutral NADES type, FS-H_2O, although sucrose hydrolysis also occurs at very concentrated sucrose solution even at neutral pH [42].
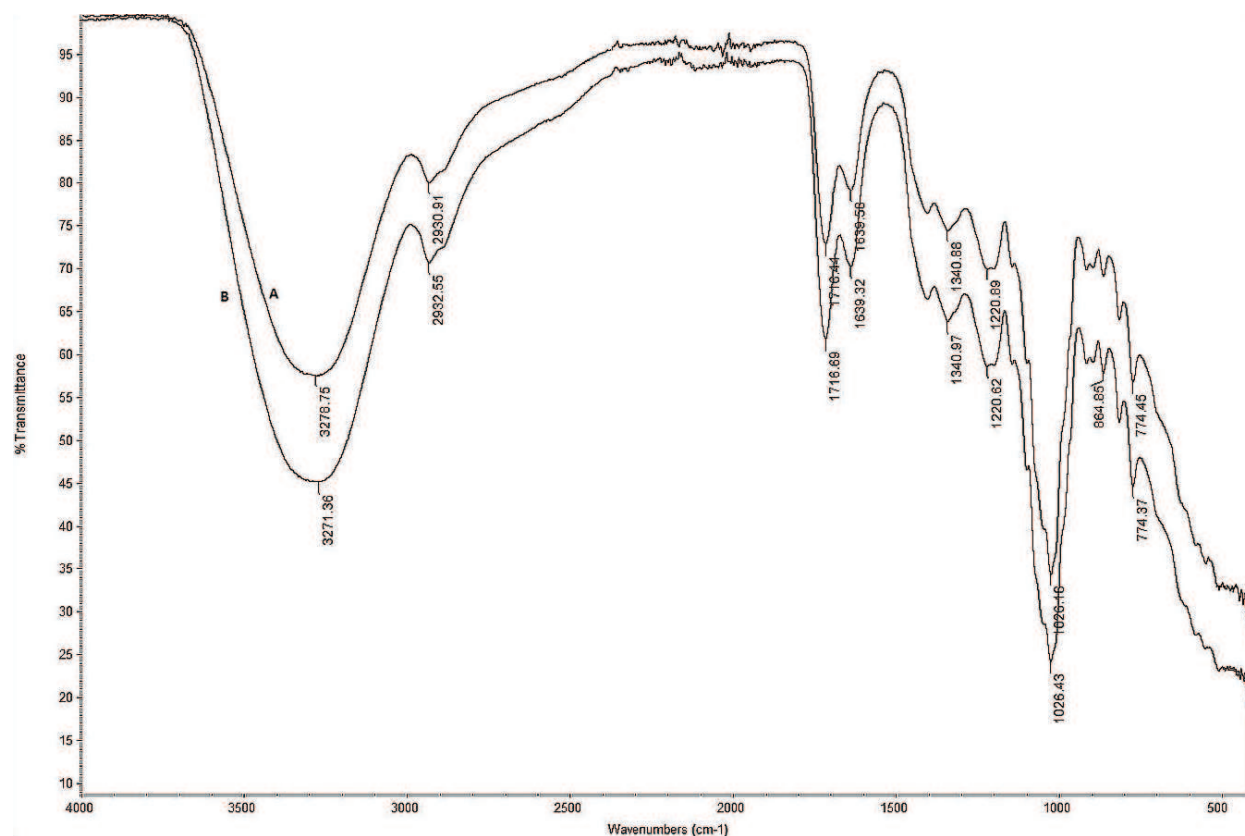
Figure 7. FT-IR spectra of CAS-H$_2$O, (A) using technical grade and (B) using pure grade of citric acid.

The resulted data of curcuminoid extraction with NADESs are shown in **Figure 9**. It is surprising that in overall NADESs show better extracting capability of curcuminoids than organic solvent, i.e. ethanol, and water although NADESs themselves are water-based solvents (**Figure 9**). Curcuminoids can be extracted by NADES due to the hydrogen bond formed between curcuminoids with NADES suggesting the presence of hydroxyl groups in curcumin, demethoxycurcumin as well as bisdemethoxycurcumin. At the same time, none of curcuminoids was extracted with both ethanol and water using the same extraction protocol as NADES. Curcumin is practically insoluble in water, i.e. ca. 4 ppb (4 µg/L at pH = 7,3) [44]. Curcuminoids were the best extracted by CCMA-H$_2$O (1:1:3), 0.355 ± 0.019 mg/g, which is in agreement with that reported by Euterpio et al. [35] and Kwon and Chung [45]. A 0.136 mg curcuminoids/g of *C. longa* was obtained using a subcritical mixture of MeOH-H$_2$O (50:50, v/v) at 135°C, 5 atm for 5 min [45], while pressurized hot water extraction (PHWE) yielded 0.503 and 0.204 mg curcuminoids/g *C. longa* at 90 and 250°C, respectively [35] although *C. longa* contains higher curcuminoids, i.e. ca. 4.4% of dry weight, than *C. zedoaria*.

The neutral, ionic and basic types of NADESs give more or less similar yields of curcuminoids, while the lowest yields were obtained by acidic types of NADESs, i.e. CAS-H$_2$O (1:2:15) and MAS-H$_2$O (1:1:11) with yields of 0.151 ± 0.001 and 0.131 ± 0.002 mg/g, respectively. Though CCMA-H$_2$O (1:1:2) yielded the highest curcuminoids, apparently there is no direct relation between solubilizing capacity of the NADES with respect to curcumin and the
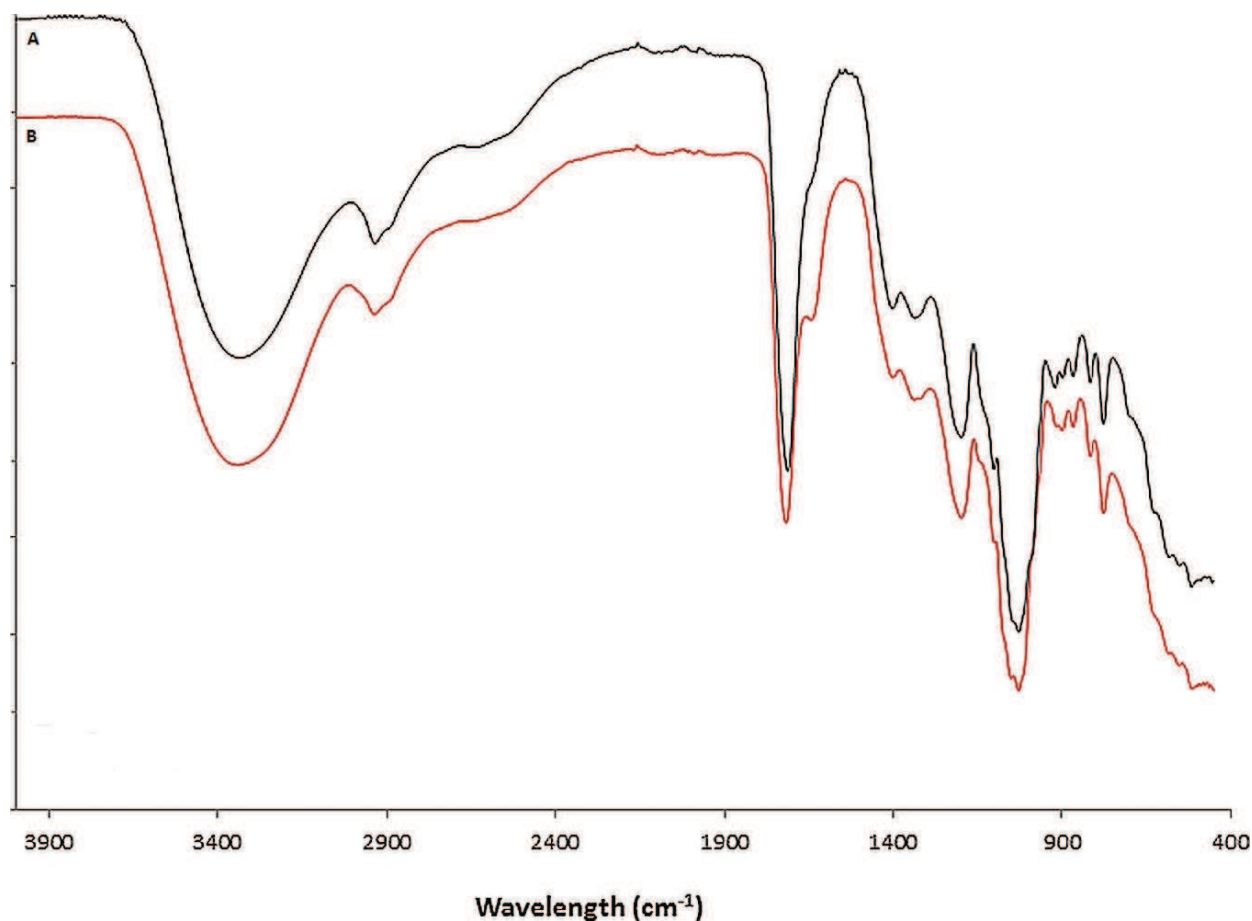
Figure 8. FT-IR spectra: (A) clear and transparent CAS-H$_2$O and (B) yellowish CAS-H$_2$O.

polarity, water content or pH [40]. However, exhaustive extraction of *C. zedoaria* using Soxhlet and maceration with ethanol (96%) as solvent only gave 0.119 ± 0.0001 and 0.152 ± 0.010 mg curcuminoids/g dry weight, respectively. Degradation of curcumin might occur at high temperature (78°C) during Soxhlet extraction. Salem et al. [46] reported that curcumin degraded after 24 h exposure at 70°C.

Longer curcuminoid extraction with CCMA-H$_2$O (1:1:2) up to 96 h (4 days) only gave a yield of 0.233 mg/g ± 0.017, i.e. approximately 36% less than that obtained after 24 h extraction. Though curcumin is reported to be stable at 10–55°C [46], prolonged exposure at 40°C (ca. 96 h) could degrade curcumin. In addition, CCMA-H$_2$O (1:1:2) could not stabilize curcumin, and extraction time may affect the obtained yield. In fact curcumin is precipitated after about 3 days due to its low water solubility at pH = 1–7 [47]. The CCMA-H$_2$O (1:1:2) had a pH of 2 at 10x dilution with Aquadest. The native pH of CCMA-H$_2$O (1:1:2) cannot be measured due to its inherent high viscosity.

In NADES with higher water content such as FS-H$_2$O (2:1:26) (water content = 40% by weight), only 37–52% curcuminoids were left after 96 h. This coincides with Tonnesen and Karlsen [47] who reported that the degradation of curcumin will be faster approximately 100x than that in concentrated solution at pH < 7. It clearly concludes that water content may affect the stabilizing
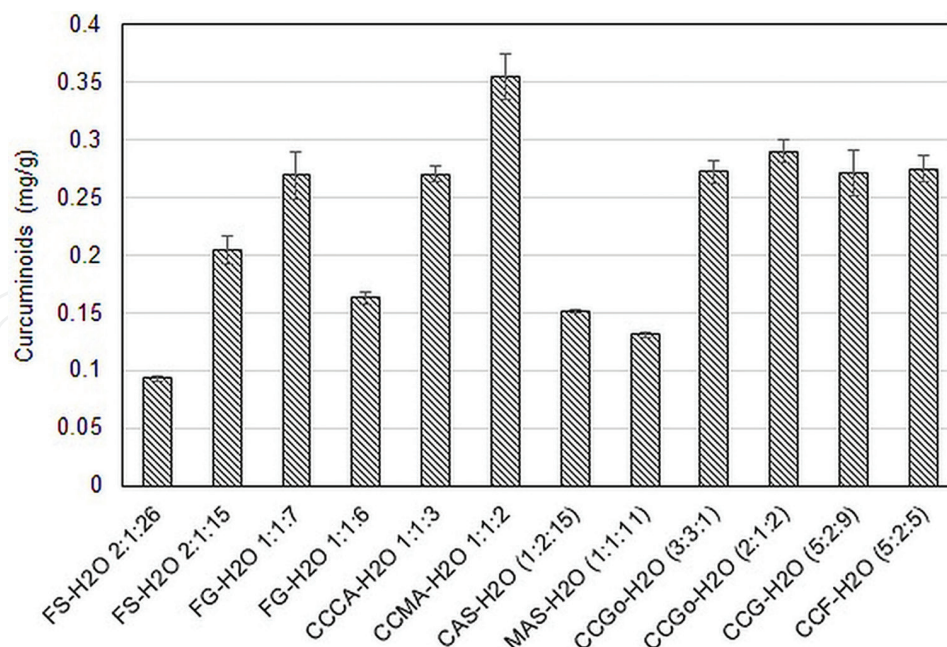
Figure 9. Curcuminoids extracted (mg curcuminoids/g dry weight) from *Curcuma zedoaria* with different types of NADES (G = glucose, F = fructose, S = sucrose, CC = choline chloride, MA = malic acid, CA = citric acid and Go = glycerol).

ability of NADES to curcuminoids. Moreover, the low level of curcuminoids might also be due to the loss of NADES structure since the hydrogen bond will rupture when NADES is further diluted with water content higher than 50% [20]. Though higher water content minimized the mass transfer resistance between NADES and plant matrix, water dilution affects considerably the target compounds and the nature of NADES itself.

A profile of extracted curcuminoids was also studied to find the selectivity of NADES to curcuminoids (**Table 4**). It is shown that NADES and FS-H_2O (2:1:26) are less selective to bis-demethoxyxurxumin (BDMC), but it is more selective to curcumin (ca. 54 wt.%) than to DMC (ca. 32 wt.%). Other kinds of NADESs also give different selectivity. The exhaustive extraction with 96% ethanol gave a similar selectivity, i.e. approximately 17, 23 and 60 wt.% for BDMC,

Extraction method	Yield (mg/g [a]) (% [b])			Extracted curcuminoids (mg/g ± SD)
	BDMC[c]	**DMC**	**C**	
Soxhlet (EtOH 96%)	0.020 (17)	0.028 (23)	0.071 (60)	0.120 ± 0.020
Maceration (EtOH 96%)	0.026 (18)	0.034 (21)	0.092 (61)	0.150 ± 0.660
FS-H_2O (2:1:26)	0.013 (14)	0.030 (32)	0.051 (54)	0.093 ± 0.002
CCMA-H_2O (1:1:2)	0.017 (5)	0.072 (20)	0.266 (75)	0.355 ± 0.019

[a]Yield expressed with mg bioactive compound/g dry weight of *C. zedoaria*.

[b]Percentage of weight to the total weight of extracted *curcuminoids* (*curcuminoid* = BDMC + DMC + C).

[c]*Bisdemethoxycurcumin* (BDMC), *demethoxycurcumin* (DMC) and *curcumin* (C).

Table 4. Profile of the extracted *curcuminoids* (mg/g) with different extraction methods.

Trial	Pressurized extraction conditions						NADES[c]	Results
	Plant material[a]	Weight (mg)	Preheat (min)	Holding (min)[b]	Temp (°C)	Press (bar)		
E1 [d]	Pwd	100	30	15	50	100	CHCA (1:1) GMA (1:1)	Clogging
E2 [d]	Frz	100	30	15	50	100	LPrS (2:1)	
E3 [d]	Pwd	300	60	2	50	50	MAS (1:1) CAS (1:1) GMA (1:1)	Clogging
E4 [d]	Pwd	200	60	2	50	50	CHCA (1:1) βAMA (1:1) LPrS (2:1)	Clogging
E5 [e]	Pwd	200	60	2	50	50	CHCA (1:1) GMA (1:1) LPrS (2:1)	Clogging
E6 [f]	Pwd	200	60	2	50	50	MAS (1:1) CAS (1:1) βAMA (1:1)	None

[a]Plant material Pwd = powder, Frz = freeze-dried. All the materials have 25–53 μm of particle size. Plant material and NADES were mixed prior loaded to the extractor cells.

[b]Heat up and discharge time is in default setting. They were 1 and 5 min, respectively. All the experiments were conducted with two cycles.

[c]Ratio is in molar.

[d]Plant material, NADES and 1 g of sea sand were mixed prior loaded to the extractor cells. There were flushing with solvent (water) and gas (N_2) between cycles, 1 min each. Around 3 g of sea sand was placed in the upper and lower part of the extractor cells.

[e]Plant material, NADES and 1 g of sea sand were mixed prior loaded to the extractor cells. No flush with solvent (only with gas 1 min) between cycles. Around 3 g of sea sand was placed in the upper and lower part of the extractor cells.

[f]Plant material, NADES and 1,5 g of sea sand were mixed prior loaded to the extractor cells. No flush with solvent (only with gas 1 min) between cycles. Around 2.5 g of sea sand was placed in the upper and lower part of the extractor cells.

Abbreviations: β-alanine (**βA**), citric acid (**CA**), choline chloride (**CH**), glucose (**G**), malic acid (**MA**), L-Proline (**LPr**), sucrose (**S**).

Table 5. Preliminary extraction of pressurized extraction for finding the best NADES extraction conditions.

DMC and C, respectively. Hence, curcuminoid selectivity depends on the types of solvent. Moreover, in the case of curcuminoid extraction from *C. zedoaria* powder, the ratio of solid to NADES also affected the yield (data not shown). In conclusion, NADES is a better solvent to solubilize curcuminoids than water and ethanol. NADES is more selective to curcumin followed by DMC, while ethanol only gave 60% selectivity to curcumin.

The extraction of an alkaloid, galantamine, with NADES from its plant matrix *Narcissus pseudonarcissus sp. was* also conducted. A preliminary and simple extraction method similar to that of *C. zedoaria* explained above was also conducted with ground powder of *N. pseudonarcissus* with particle size around 25–53 μm. However, none of the galantamine was extracted although the extraction time was prolonged up to a week. This is probably because the mechanical stirring could not overcome the mass transfer resistance around the pellet of *N. pseudonarcissus* due to the structure of plant matrix.

Therefore, a pressurized extraction method using a pressurized extractor apparatus E-916 (Büchi, Flawil, Switzerland) was performed. It is a fast, simple and reproducible method facilitated by high-pressure condition instead of mechanical agitation. In addition, it is also a fast screening method to find the best extraction condition for NADES extraction. Preliminary experiments were conducted to find the best extraction configuration, whilst NADESs are used as a solvent as shown in **Table 5**.

Two different kinds of plant matrix were used, i.e. ground powder and freeze-dried powder of *N. pseudonarcissus* bulb. Both have particle sizes of 25–53 μm. NADES with mild viscosity was chosen to minimize clogging problem inside the extraction cell which is essentially a packed bed where the bulk porosity is important to make a good contact between NADES and plant matrix. If the sample inside the extraction cell is too compact, it creates a clogging problem and overpressurized the cell; otherwise the contact between the solute and solvent will be minimal leading to a low yield.

At high-pressure conditions (100 bar), E1 and E2, NADESs were overcooked, and the sugar was caramelized although only 30 min of preheating time was applied. Therefore, the extractions were performed at a lower pressure (50 bar). To compensate the pressure reduction, the preheating time was increased. Finally, extraction condition E6 was found to be free of clogging and overcooked problems. Further extraction of galantamine with NADES by applying E6 condition gave 6.11 and 9.33 mg of galantamine/g dry weight for CAS (1:1) and MAS (1:1), respectively. These yields were higher than that obtained by water extraction as a control (5.35 mg of galantamine/g dry weight). These values were also higher than galantamine extraction with supercritical CO_2, i.e. 303 μg/g dry weight (70°C, 220 bar, 3 h) [48]. The selectivity of NADES to galantamine was slightly better (70–78%) than that of SC-CO_2 (<70%). Thus, NADES extraction of galantamine is more efficient, in terms of both galantamine yield and selectivity.

5. Concluding remarks

Deep eutectic solvents (DESs), including natural deep eutectic solvents (NADESs), are new generation of solvents. DES can be prepared by mixing two or more components at eutectic composition so that the mixture has lower melting point than those of the constituent components. DESs are typically prepared from quaternary ammonium halide salts and hydrogen bond donors (HBDs), such as amide, carboxylic acid, alcohol, sugar, amino or organic acid and alkylsulfate or alkyl phosphate. The behaviors and properties of DESs can be adjusted by varying the hydrogen bond donors and their molar ratio in the mixtures [13]. The polarities of DESs can match those of conventional organic solvents; however, DESs have several advantages, such as non-toxic, non-volatile, inflammable and biodegradable. Therefore, DESs can be considered as green solvents.

DESs potentially have unlimited number of applications. The applications of DESs to purify crude biodiesel made from rice bran oil and to extract natural bioactive compounds, such as oryzanol, cucurmin and galantamine, have been discussed in this chapter. DESs could give higher extraction yields of natural bioactive compounds than those obtained using conventional

organic solvents. Besides that, the use of DESs could lead to a simpler extraction or separation procedure, especially in a large industrial scale, due to non-toxic and inflammable nature of DES.

However, at the same time, one has to consider that there is no universal solvent for all kinds of compounds. Each material and targeted compound requires the development of a specific process. No single standard procedure of extraction is suitable for extractions of all secondary metabolites. This also allows a rather selective extraction which is particularly of interest for the isolation of pure compounds. Some parameters that should be considered in the development of a DES-extraction procedure are types of the matrix plant, type of extraction process, ratio of plant material to solvent and extraction conditions: temperature, duration of extraction, viscosity and water content of DES.

Author details

Siti Zullaikah[1]*, Orchidea Rachmaniah[1], Adi Tjipto Utomo[1], Helda Niawanti[2] and Yi Hsu Ju[3]

*Address all correspondence to: szulle@chem-eng.its.ac.id

1 Department of Chemical Engineering, Institut Teknologi Sepuluh Nopember, Kampus ITS Keputih Sukolilo, Surabaya, Indonesia

2 Department of Chemical Engineering, Mulawarman University, Samarinda, Indonesia

3 Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan

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