Natural deep eutectic solvents and their application in natural product research and development

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Chapter 6

Natural Deep Eutectic Solvents facilitating the extraction and storage of anthocyanins

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Abstract

Natural deep eutectic solvents (NADES) have a high solubilizing capacity, which is promising for their application in the extraction of active compounds from herbal medicines. Natural deep eutectic solvents are composed of neutral, acidic or basic compounds that form liquids of high viscosity when mixed in certain molar ratios. The viscosity may affect the extraction properties and stabilizing ability of NADES as well as the chromatographic properties of the extracts obtained. To address these problems, as a model, extraction methods with NADES were investigated in combination with HPLC-UV based metabolomics for the analysis of anthocyanins in flower petals of *Catharanthus roseus*. Stability tests of the anthocyanin extracts were also done. Multivariate data analysis indicates that NADES made of lactic acid-glucose (LGH), and 1,2-propanediol-choline chloride (PCH) present similar extractability of anthocyanins as acidified methanol. Furthermore, LGH exhibits higher stabilizing ability for cyanidin than acidified ethanol. Cyanidin was stable for 7 days at 4 ℃ and 3 month at -20 ℃ in LGH in the dark, which facilitates their extraction and analysis process. Compared with conventional organic solvents, NADES provide a greener and more stable approach for the extraction and storage of anthocyanins, implying that they have the potential to replace conventional organic solvents in health related areas such as food, pharmaceuticals, and cosmetics.

Key words: Natural deep eutectic solvent; anthocyanins; extractability; stabilizing ability; chromatographic behavior; *Catharanthus roseus*.

1. Introduction

A new type of green solvents, the natural deep eutectic solvents (NADES) were proposed in our group (Choi *et al*., 2011; Dai *et al*., 2013). NADES are liquid supermolecules made of natural primary metabolites (e.g. sugars, sugar alcohols, organic acids, amino acids, and amines) held together by intermolecular interactions, particularly H-bonding. NADES present many advantages for extraction (Dai *et al*., 2013), e.g., negligible volatility, adjustable viscosity, and high solubilization strength. For example, some NADES show a very high solubilization ability for rutin, as much as 12,000 times higher than water (Choi *et al*., 2011; Dai *et al*., 2013). From an environmental and economic perspective, NADES also present great advantages including their biodegradability, sustainability, low cost, and simple preparation. All those properties indicate their great promise as good extraction solvents for natural products and their potential applications in health related areas such as food, pharmaceuticals and cosmetics.

The structure of NADES and relevant physicochemical characteristics are to be considered. The major components of NADES have several functional groups such as hydroxyls, carboxyl or amino groups (Choi *et al*., 2011; Dai *et al*., 2013). Those groups can interact forming H-bonds between the components, leading to highly structured viscous liquids, which account for their special physical properties and different solvent behavior compared to conventional solvents. Those liquids can also form hydrogen bonds with solutes, thus greatly increasing the solubility of compounds in NADES, e.g. phenolic compounds. NADES can be divided into five groups according to the nature of their components: ionic liquids with an acid and a base, NADES with neutral compounds, sugar-based NADES with an acid, sugar-based NADES with a base and sugar-based NADES with an amino acid (Dai *et al*., 2013). The different compositions of NADES result in their broad range of physical properties, probably leading to different behavior in applications such as extraction, analysis and storage of natural products. Therefore, further studies are required to develop diverse applications of NADES, such as the extraction of herbal medicines.

Anthocyanins are a widespread group of natural water soluble phenolic compounds that occur in plants. They exist mainly in flowers, fruits, and vegetables, being responsible for a great part of the orange, red, purple, and blue colors (Castañeda-Ovando *et al*., 2009). Since the 1990s, there is a renewed interest in anthocyanins because of their possible health benefits as antioxidant and anti-inflammatory agents, especially for the prevention of diseases such as cardiovascular diseases and cancer (Wang *et al*., 1999; Noda *et al*., 2002; Hou, 2003; Kong *et al*., 2003; Albrecht *et al*., 2004). Plant extracts containing anthocyanins, such as pomegranate extract, have also been developed as "functional foods" and "herbal/ nutritional supplements" in developed countries (Ismail *et al*., 2012). In plants, anthocyanins are almost always found in the form of glycosides with different sugar substituents, and different aliphatic or aromatic carboxylic acids bonded to sugar units (Kong *et al*., 2003). There are six major

aglycones (anthocyanidins): delphinidin, cyanidin, pelargonidin, petunidin and malvidin, differing only in the number and position of hydroxyl or methoxyl substitutions in the B-ring.

The general solvents for the extraction of anthocyanins are typically mixtures of water with ethanol, methanol, or acetone (Kähkönen *et al*., 2001; Kalia *et al*., 2008). The instability of anthocyanins causes many inconveniences for their extraction, preparation, analysis process and storage (Melgarejo *et al*., 2011; Oidtmann *et al*., 2012). The anthocyanin extracts or isolated anthocyanins are highly instable and very susceptible to degradation due to temperature, light andsolvent among other factors (Giusti and Wrolstad, 2003; Castañeda-Ovando *et al*., 2009). Thismake the protocols for sample preparation and analysis of anthocyanins complex, and the whole process can often be very time-consuming. (Mazza, 2004; Dai *et al*., 2009; Navas *et al*., 2012). In general, it is recommended that the extraction of anthocyanins be performed in the dark at a low temperature (Awika *et al*., 2005; Piovan *et al*., 1998). Therefore, it is very useful to develop new green solvents that combine high extractability and stabilization ability for anthocyanins.

In this study, the extractability and storage stabilization properties of some typical NADES for anthocyanins were explored. A simple extraction method for anthocyanins with NADES combined with HPLC/UV-based metabolomics for anthocyanins was designed. As a model, *Catharanthus roseus* petals of purple and orange color were selected because they contain different kinds of anthocyanins. The stability of cyanidin in NADES was investigated considering the effects of temperature, light and storage time. The physical properties of NADES which may affect the extraction, analysis, and storage process are discussed, laying the basis for their further applications.

2. Materials and methods

2.1 Chemicals and materials

Two different flower-colored *Catharanthus roseus* varieties were purchased from Intratuin, Pijnacker (Postbus 1016, 1700BA, Heerhugowaard, The Netherlands), belonging to the Pacific series i.e. Pacifica XP Apricot (orange color), and Pacifica Orchid Halo (purple, color) were used in this study. Flower petals were collected, ground to powder with liquid nitrogen and freeze dried in September 2009 during the blooming season of *C. roseus*. Methanol and ethanol of analytical grade and methanol of HPLC grade were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was of deionized water quality. Malic acid, lactic acid, proline, glucose, fructose, sucrose, 1.2-propanediol, and choline chloride from Sigma (St. Louis, MO, USA) and cyanidin standard (Carl Roth, Karlsruhe) were used.

2.2 Natural deep eutectic solvents and solution preparation

All NADES including 1,2-propanediol-choline chloride (PCH); lactic acid-glucose (LGH); proline-malic acid (PMH); malic acid-choline chloride (MCH); glucose-choline chloride (GCH); glucose-fructose-sucrose (FGSH) were prepared (Dai, *et al*., 2013). Proline aqueous solution (65 mg/ml) and malic acid aqueous solution (50 mg/ml) were prepared with deionized water.

2.3 Extraction methods optimization

*Heating and stirring***.** Plant material (50 mg) with 1.5 mL NADES or methanol with 3% formic acid was stirred at 40 ^{o}C for 30 min in a sealed glass bottle. The sample was transferred into an eppendorf tube, centrifuged at 1300 rpm for 20 min and then filtered through a 0.45 μm cellulose acetate filter and diluted to double the volume with 3% aqueous formic acid. All extractions were performed in triplicate.

Ultrasound-assisted extraction (UAE). Plant material (50 mg) was extracted with 1.5 mL solvent in an ultrasonicator bath at room temperature for 30 min. Ultrasound-assisted extraction with heating (UEH) was carried out in an ultrasonicator bath at 40 ^{o}C for 30 min. The extract was centrifuged, filtered, and diluted as described above.

2.4 Stability test

Solutions of cyanidin reference standard in LGH (0.1 mg/mL) and ethanol with 3% formic acid (0.1 mg/mL) were used in the following stability tests. For thermal stability, cyanidin solutions in tubes with screw-caps were placed in a preheated water bath at 80 ^{o}C, 60 ^{o}C and 40 ^{o}C, respectively. Three tubes of each group were removed from the water bath at 10, 20, 40, 60, 80, 100, 120 min and rapidly cooled to room temperature. The stability of cyanidin solutions stored for three months at -20, 4 and 25 ^{o}C in the dark was evaluated by analyzing three tubes of each group at day 1, 3, 7, 15, 30, 60, 90. For the effect of light, cyanidin solutions were exposed to sunlight or kept in the dark in ambient conditions and three tubes of each group were removed at day 1, 3, 7, 15. All experiments were done by triplicate.

2.5 Apparatus and analysis

HPLC analysis was carried out on a Agilent chromatographic system with Phenomenex Luna C18(2) column (4.6 μm x 250 mm, 5 μm) at 35 degree. The mobile phase consisted of water with 3% formic acid (A) and methanol with 3% formic acid (B) in a linear gradient program as follows: 20-25% B (0-30 min), 25-45% B (30-35 min), 45-80% B (35-40 min) at a flow rate of 1.0 mL/min. Chromatograms were record at 520 nm. The injection volume was 10 μL. A UV-Vis spectrophotometer (Shimadzu, Japan) was used for measuring the stability test at a wavelength of 520 nm.

2.6 Data analysis

Calculation of kinetic parameters of cyanidin degradation at high temperature: the first-order reaction rate constants (k) and half-lives ($t_{1/2}$) were calculated by the following equation (Kırca *et al*., 2007):

$$\ln(C/C_0)=-kt$$

$t_{1/2}$=-In(0.5)/k

where $C/C_{0=}$ $A/A_{0,}$ C_0 and A_0 is the initial concentration and absorption of diluted carthamin, and C and A is the concentration and absorption value of diluted carthamin after heating time (t) at a given temperature, respectively.

The integrated areas of peaks of anthocyanins in HPLC-UV chromatograms from three replicates were analyzed with the SIMCA-P software (V. 12, Umetrics, Umeå, Sweden) for PCA analysis using the Pareto scaling method. Analysis of variance (ANOVA) was performed in SPSS software (version 14.0, Chicago, IL, USA) using the integration areas of the peaks in the HPLC chromatograms at 520 nm with P values ≤ 0.05 considered as significant.

3 Results and discussion

3.1 Comparing the extraction of anthocyanins with NADES and methanol

Catharanthus roseus has been wellstudied in our lab for many years (Mustafa *et al*., 2007). They have petals with more than 5 different colors, e.g. pink, light pink, purple, red, and orange. Higher amounts of hirsutidin 3-*O-(6-O-p-coumaroyl)* glucose and petunidin 3-*O-(6-O-p-coumaroyl)* glucose, an intermediate level of malvidin 3-*O-(6-O-p-coumaroyl)* glucose, and a low level of petunidin 3-*O*-glucose and trace amounts of some others were observed in *C. roseus* (Piovan *et al*., 1998). The anthocyanin profiles in *C. roseus* are different for the 5 different flower colors and those petals of the purple and orange flowers exhibit more peaks (6-9) at the wavelength of 520 nm than the other colors, as shown in a previous study (Fig. *S*1, unpublished). The purple- and orange-colored petals of *C. roseus* were selected to investigate the extractability of NADES for anthocyanins.

The HPLC fingerprints at 520 nm show the same qualitative chemical profiles of anthocyanins in some tested NADES and methanol with 3% formic acid extracts. Nine peaks were observed for the purple petal extracts (Fig. 2a) and 6 for the orange petals (Fig. 2b). To quantify the different extractability of NADES and methanol for anthocyanins, multivariate data analysis (PCA) was applied with the peak areas of all peaks at 520 nm as variables. PCA is one of the most widely used multivariate data analysis methods to reduce the dimensionality of a multivariate dataset. The PCA gives a score plot of principle components, which can be used to identify the separation and similarities among all the analyzed samples. The variables responsible for the separation or grouping among samples are plotted in a loading plot and variables with a high value far from the center are most important. The score plot of PCA (Fig. 3a) with the first two components (R^2 =0.94 and Q^2 =0.67) shows a separation of the extracts from purple petals into 2 groups, separated by PC1. The LGH, PCH, 75% FGSH and acidified methanol extracts are in the positive area of PC1 (group I) and PMH, MCH, GCH are in the negative part of PC1 (group II). The score plot implies that LGH and PCH have similar extraction characteristics to acidified methanol for anthocyanins from the purple flowers of *C. roseus*. The loadings plot (Fig. 3b) shows that all anthocyanins peaks are located in the positive area of PC1 (group I), indicating that solvents in group I

(PGH, LGH, 75% FGSH and acidified methanol) are better solvents than those in group II (PMH, MCH and GCH). Methanol with 3% formic acid was selected as a reference because of its high extraction yield of anthocyanins (Metivier *et al*., 1980; Awika *et al*., 2005). Both LGH and PCH are suitable extraction solvents for anthocyanins, being as efficient as acidified methanol.

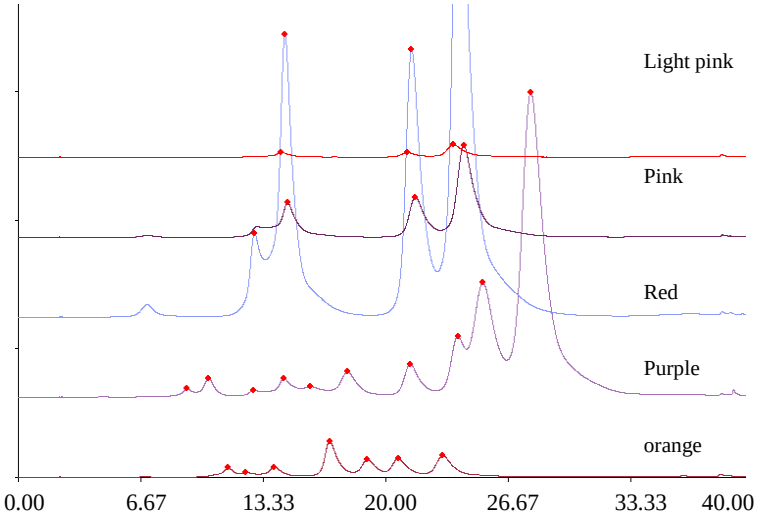

Fig. 1. HPLC-UV chromatograms at 520 nm of anthocyanins from *Catharanthus roseus* with different flower colors extracted with methanol 3% formic acid.

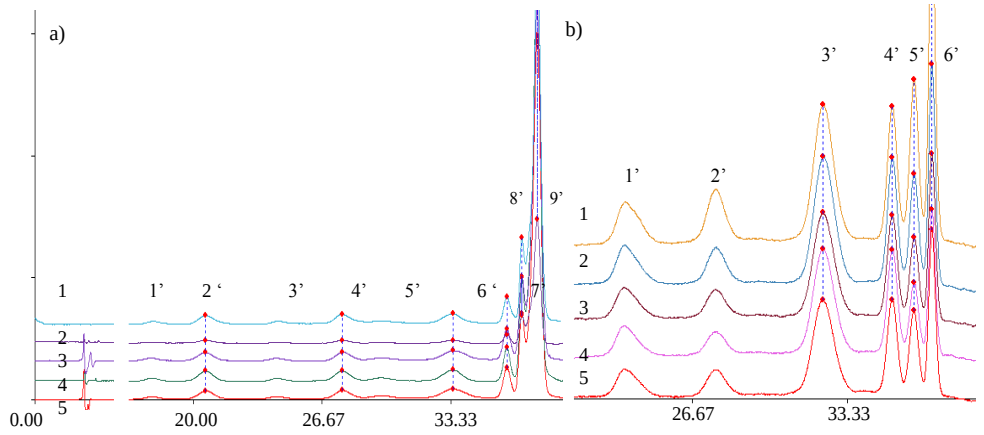

Fig. 2. HPLC-UV chromatograms of anthocyanins from *Catharanthus roseus* with **a**) purple and **b**) orange flower petals extracted with different solvents at 520 nm. (**1**) methanol; **2**) lactic acid-glucose(LGH); **3**) malic acid-choline chloride(MCH); **4**) prolinel-malic acid(PMH); **5**) glucose- choline chloride(GCH)).

For the anthocyanins in the orange-colored petals of *C. roseus*, a similar separation model was observed. The score plot of PCA with the first two components (R^2 =0.95 and Q^2 =0.82) shows that all the extracts were separated into two groups by PC1 (Fig. 3c), with acidified methanol, LGH, PCH in the positive

area of PC1 (group I) and PMH, MCH, and GCH in the negative area of PC1 (group II). The loading plot (Fig. 3d) shows that all peaks of anthocyanins are located in the positive area of PC1, indicating that solvents in group I give higher extraction yields than solvents in group II.

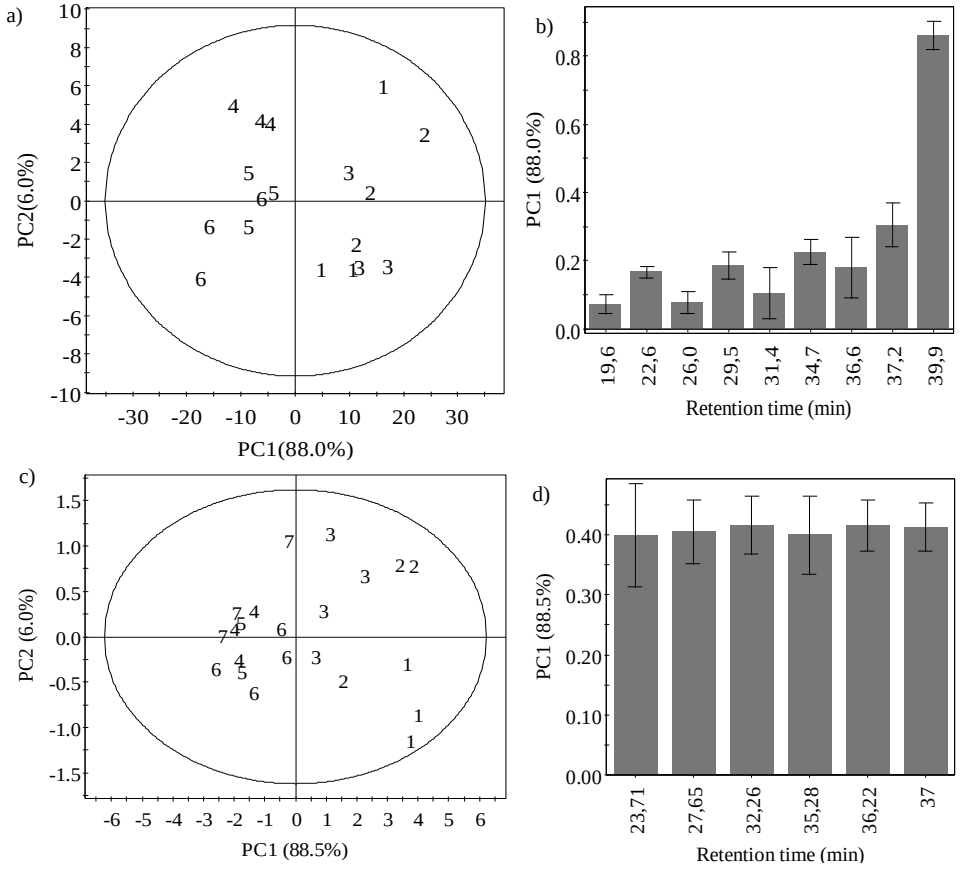

Fig. 3. Score plots (**a**, **c**) and loadings plots (**b**, **d**) of principal component analysis of extracts of *Catharanthus roseus* flowers with purple (a,b) and orange color (c,d) by different solvents (methanol 3% formic acid (**1**); 1,2-propanediol-choline chloride-water (**2**); lactic acid-glucose-water (**3**); proline-malic acid (**4**); malic acid- choline chloride (**5**); glucose-choline chloride (**6**); 75% glucose-fructose-sucrose (7).

A further ANOVA test on the average peak area of anthocyanins in HPLC-UV chromatogram confirms that LGH and PCH have the same extraction yields of both colored anthocyanins as acidified methanol, which is more efficient that the other tested NADES. ANOVA shows that the area of the peak (retention timeat 39.9 min) is significantly higher in LGH, PCH, and acidified methanol than in the others (Fig. 4), which confirms the results from PCA that LGH, PCH, 75% FGSH

possess similar solvent strength as acidified methanol and much higher than other NADES.

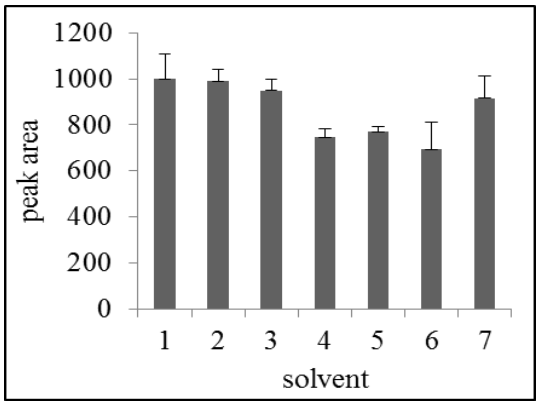

Fig. 4. The average peak area of peak (retention time at 39.9 min) of anthocyanins extracted from purple-colored petals of *Catharanthus roseus* with different solvents (the same as figure 3) in HPLC chromatogram at 520 nm. The data is expressed in mean ± SD (*n*=3).

The high extraction yield of PCH and LGH may be related to their physicochemical properties. To test the effect of viscosity, polarity, and chemical composition of NADES on their extracting characteristics, the following 6 typical NADES with different physical properties were selected for extraction: PCH, LGH, PMH, MCH, GCH, and FGSH (Dai *et al*., 2013). In the first place, the high extraction yields of anthocyanins with LGH and PCH are correlated with their low viscosity. PCH, LGH (ca. 35 mm^2/s) have the lowest viscosity among all the tested NADES, GCH (397 mm^2/s), PMH (251 mm^2/s), MCH (446 mm^2/s) (Dai *et al*., 2013). In the case of FGSH it was diluted with 25% volume of water for use, resulting in a similar viscosity as water (1 mm^2/s). Secondly, the solvent strength of NADES seems to have no direct relationship with their polarity. In the case of conventional solvents, the extraction efficiency can be estimated by polarity. However, the NADES do not show a relationship between the polarity and extraction yield. LGH and MCH have the highest polarity (44 kcal/mol), followed by PMH (48 kcal/mol), sugar/sugar alcohol-choline (49 kcal/mol); PCH has the lowest polarity (50 kcal/mol) (Dai *et al*., 2013). Lastly, no relationship between the acid or base components of NADES and their extraction capacity was observed. One method to increase the extractability of anthocyanins in conventional solvents is to add a small amount of acid such as acidified methanol (Awika *et al*., 2005). The pH values of aqueous NADES are different when diluted with 90% (v/v) of water due to the acid or base components in NADES (Dai *et al*., chapter 4). LGH and PCH with the same extractability have different pH values, while LGH and MCH have the same pH but different extraction yields of anthocyanins. All considered, as opposed to conventional solvents, the extraction efficiency of NADES is likely to be more correlated to the viscosity of NADES rather than their polarity and pH values.

3.2 Optimizing extraction and analysis method for anthocyanins with NADES

*3.2.1 Extraction methods optimization.*Three extraction methods were examined for the extraction of anthocyanins from flower petals of *C. roseus*. The extraction yield increased by 35-55% on stirring at 40 ^{o}C and only by 2-20% with sonicating at 40 ^{o}C if compared with sonication at 25 ^{o}C (Table 1). Extraction times between 30 and 90 min were examined using the stirring method at 40 ^{o}C. The result shows there is no difference in extraction yields with 30 and 60 min extraction and at 90 min the peak areas of all peaks in fact decreased (Table. 2). Long extraction time allows the dissolution of compounds but may also lead to the formation of artifacts or degradation of compounds. Thus, stirring at 40 ^{o}C for 30 min was selected for this study.

Table 1. Influence of three different extracting conditions on the relative extraction yield of anthocyanins from *Catharanthus roseus* with purple petals.

	Relative extraction yield as peak area ratio[a]		
peaks	**sonication (25 ℃)**	**sonication (40 ℃)**	**stirring (40 ℃)**
peak 1	0.6	0.8	1.0
peak 2	0.5	0.6	1.0
peak 3	0.6	0.4	1.0
peak 4	0.5	0.5	1.0
peak 5	0.4	0.3	1.0
peak 6	0.6	0.6	1.0
peak 7	0.5	0.7	1.0
peak 8	0.5	0.7	1.0
peak 9	0.5	0.7	1.0

[a]The relative extraction yield is expressed in peak area ratio of the peaks in the chromatogram of the extract as compared to the reference (40 ℃, stirring) (n=3).

Compared with conventional solvents, a disadvantage of NADES is their high viscosity, which causes slow mass transfer and decreases their extractability. In order to improve this, the above three approaches were explored with the purpose of decreasing the viscosity and improving extraction efficiency. Our previous study showed that increasing temperature leads to a decrease in viscosity of NADES (Dai *et al.*, 2013), but a high temperature can cause the degradation of anthocyanins (Kırca *et al.*, 2007). Thus, a mild temperature of 40 ^{o}C was selected. The second way to increase mass transfer and to speed up the diffusion of compounds in NADES is applying external forces such as stirring and sonication. Stirring is the simplest way to speed up the transfer rate of the compounds in the liquid. Ultrasonication combined with mild heating might be a good choice to assist extraction with NADES due to the acoustic cavitation phenomenon that leads to a disruption of cell walls causing the release of cellular contents and breaking

intermolecular interactions and therefore speeding up dissolution (Vinatoru, 2001). It has the following advantages: no limitations for solvent selection, high extraction efficiency within 30 min, and mild temperature operation favorable for thermally unstable compounds. Ultrasonication-aided extraction is widely used in the extraction of metabolites from plant materials (Ajila *et al*., 2011; Chen *et al*., 2007). However, with NADES as extraction solvent, sonication proved to be less efficient than stirring due to the high viscosity of NADES.

Table 2. Relative extraction yield of the peaks of anthocyanins from *Catharanthus roseus* with red petals at different times (30, 60 and 90 min).

	relative extraction ratio as peak area ratio[a]		
	30 min	**60 min**	**90 min**
peak 1	1.00	1.04	0.88
peak 2	1.00	1.01	0.99
peak 3	1.00	0.96	0.93
peak 4	1.00	0.97	0.95

[a]The relative extraction ratio is expressed in peak area ratio of the peaks in the chromatogram of the extract as compared with the reference (30 min) (n=3) .

3.2.2 The compatibility of NADES with reversed-phase HPLC. The chromatographic behavior of malic acid and proline in aqueous solutions and their deep eutectic mixture (PMH) were compared (Fig. 5). The same retention time of malic acid in aqueous solution and PMH was observed, and the same applied to proline. The chromatographic behaviors of malic acid in two NADES (PMH and MCH) diluted 1, 2 or 3-fold with water were compared with that of an aqueous solution of malic acid (Table 3). The same retention time and peak shape of malic acid were observed in the different dilutions of NADES, as in the aqueous solution. These results indicate that the interaction between the two components of NADES with at least 50% (v/v) water has no effect on the chromatographic behavior of components of NADES (malic acid). This is probably due to the fact that the interaction within the components of NADES could weaken with water dilution and even break completely with up to 50% (v/v) water (Dai *et al*., 2013; Gutiérrez *et al*., 2010). Additionally, the chromatographic behavior of anthocyanin extracts in methanol and different NADES were also compared. The anthocyanin extracts have the same profiles (considering the retention time and peak shape) in NADES as in methanol, and NADES with different compositions (Fig. 1), indicating that components of NADES have little effect on the chromatographic profiles of anthocyanin extracts in this study. In conclusion, it is recommended to dilute NADES extracts with 2 volumes of water for their analysis.

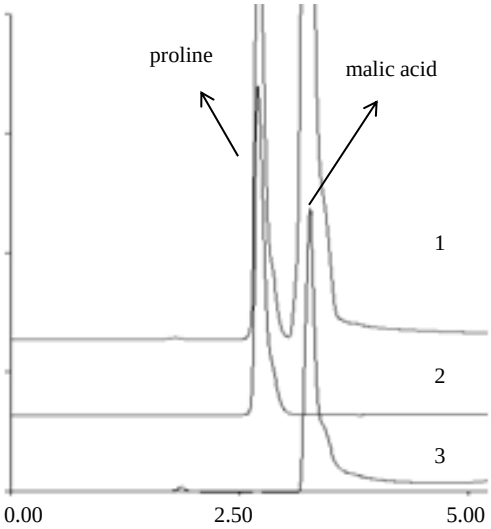

Fig. 5. HPLC-UV chromatograms of **1**) proline-malic acid-water (1:1:3) (PMH) diluted with same volume of water; **2**) proline aqueous solution; and **3**) malic acid aqueous solution at 210 nm.

Table 3. Retention time (Rt) and symmetry of the peak of malic acid in water solution (0.3 g/mL), malic acid-choline chloride-water (1:1:2) (MCH), and proline-malic acid-water (1:1:3) (PMH) with different water dilution (2-5 times) in HPLC-UV chromatograms (*n*=3).

solvents (dilution time)	Rt	symmetry
malic acid aqueous solution	4.15	0.58
MCH(2)	4.15	0.57
MCH(3)	4.15	0.57
MCH(5)	4.13	0.58
PMH (2)	4.13	0.58
PMH (3)	4.14	0.57

Regarding the chromatographic system, it is also recommendable to start with a low amount of organic solvents in the mobile phase as the initial composition of the mobile phase in gradient elution may affect the peak shape of solutes. Starting with a high amount of organic solvent will result in poor peak shape (data no shown). To ensure good chromatographic profiles of the analytes, it is thus better to set the ratio of the starting mobile phase with a high percentage of water. NADES are more polar than methanol. A high ratio of water will dilute the NADES and their hydrophillic ingredients will not be bound to the nonpolar reversed phase column, thus eluting unretained from the column.

3.3 Stability of anthocyanins in NADES

Stability of solutes should be considered during extraction and analysis, as well as during sample storage. Therefore the effects of solvent, temperature, storage time and light on the stability of anthocyanins in NADES were investigated using cyanidin in LGH as a model. To avoid the toxicity and volatility of methanol, ethanol was used in stability tests. Figure 6a-c shows the degradation curve of cyanidin at high temperature (40-80 °C) in LGH and ethanol with 3% formic acid. Cyanidin is more stable in LGH than in acidified ethanol at 60 °C and stable in both solvents at 40 °C for 1.5 hours in the dark. The degradation of anthocyanins over time follows a first-order reaction model at 60 °C and 80 °C, which is in agreement with a previous report on the degradation of anthocyanins (Kırca *et al*., 2007). The half-live time ($t_{1/2}$) of cyanidin was more than 3 times longer in LGH than in acidified ethanol at 60 °C (Table 4) and even longer at 80 °C in LGH than at 60 °C in acidified ethanol. Cyanidin is thus much more stable in LGH than in acidified ethanol at high temperature in the dark.

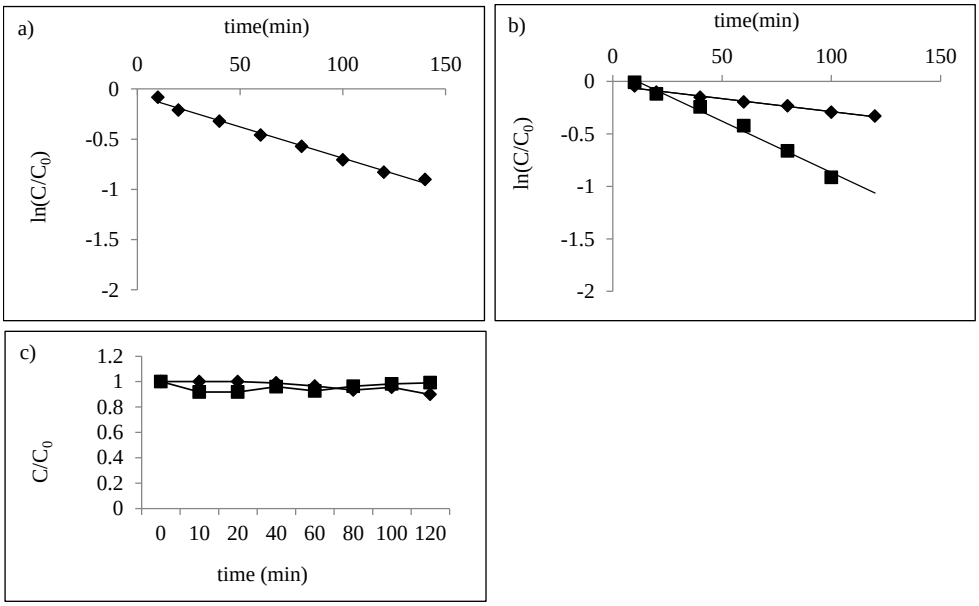

Fig. 6. Stability of cyanidin in lactic acid-glucose (LGH ♦) and ethanol with 3% formic acid (■) at **a**) 80 °C, **b**) 60 °C and **c**) 40 °C in the dark (*n*=3).

The effect of storage time and temperature on the stability of cyanidin was investigated at 25, 4 and -20 °C in the dark, both in LGH and ethanol with 3% formic acid (Fig. 7a). The different degradation curves show that cyanidin was stable at -20 °C in LGH and much more stable in LGH than in ethanol, with a similar degradation curve in LGH at 4 °C as in acidified ethanol at -20 °C. Cyanidin kept the same absorbance in LGH at -20 ℃ for 3 months, at 4 ℃ for 7 days, but degraded quickly at 25 °C, with a decrease of ca. 60% at 25 °C in 3 months, indicating that the stability of cyanidin in LGH is correlated with the temperature in the dark. Thus, samples should be stored at 4 °C in the dark and

analyzed within one week after preparation. For long-term storage, the sample can be kept at -20 ^{o}C in the dark for 3 months.

Table 4. Degradation kinetics parameters of cyanidin in lactic acid-glucose (LGH) and ethanol with 3% formic acid at high temperature including reaction rate constants (k) and half-lives ($t_{1/2}$), and the degradation functions (n=3).

t (^{o}C)	solvent	k	R^2	$t_{1/2}$ (min)	function
80	LGH	0.0062	0.9921	111.80	y = -0,0062x - 0,0653
60	LGH	0.0025	0.9844	277.26	y = -0.0025x – 0.041
	EtOH	0.0096	0.9389	72.20	y = -0.0096x + 0.1241

The effect of light was investigated at ambient conditions with sunlight compared with a control kept in the dark (Fig. 7b). In the darkness , nearly 20% cyanidin was degraded in both LGH and ethanol on day 7 while nearly 30% cyanidin in ethanol solution and 80% in the LGH solution exposed to daylight was degraded. Thus, cyanidin degrades much faster in the sunlight than in the dark both in LGH and ethanol, showing a great effect of sunlight on the stability of LGH solutions of cyanidin. Thus, cyanidin solutions should be preserved in the dark.

The higher stabilization ability of LGH for cyanidin may be correlated with the interactions between cyanidin and the molecules of LGHs. The two components of LGH may form intermolecular interactions, mainly hydrogen bonding, with cyanidin through carboxyl groups and hydroxyl groups, as in the case of quercetin in XoCH (Dai *et al*., 2013). This interaction decreases the movement of solute molecules, reduces its contact time with oxygen at the interface of NADES and air, and consequently reduces oxidative degradation, the major degradation step. It was reported that adding organic acids (such as acetic acid, citric acid, tartaric acid), sugars (such as glucose, fructose, sucrose, trehalose), and their mixtures increase color stability for anthocyanins, which is attributed to the intermolecular association (Hubbermann *et al*., 2006; Kopjar and Piližota, 2011). Cyanidin was more stable in a gel model system with pectin than with the aluminium ion, in which the stability is due to the anthocyanin-metal interaction (Buchweitz *et al*., 2012). Similarly, manually squeezed juice exhibited higher color stability than juice prepared from a concentrate, which was proposed to be due to the retention of polymeric matrix compounds in fresh juice and the interaction of this matrix with anthocyanins (Sadilova *et al*., 2009). Thus, the formation of interactions with other molecules improves the color stability of anthocyanin.

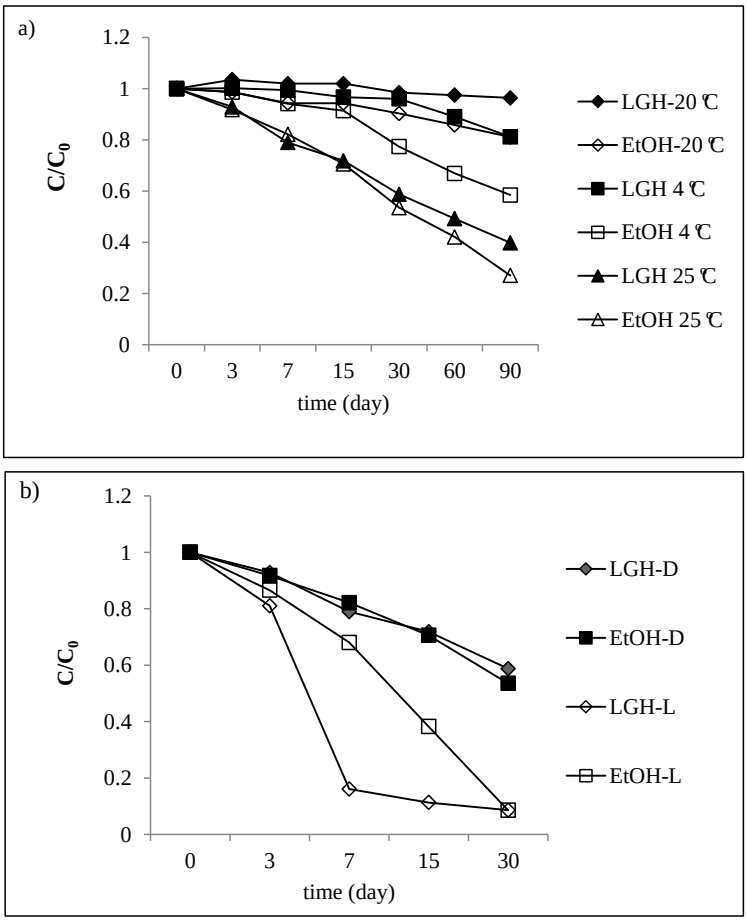

Fig. 7. Storage stability of cyanidin in lactic acid-glucose (LGH) and ethanol with 3% formic acid at **a**) -20 ℃, 4 ℃ and 25 ℃ in the dark for 3 months and **b**) at 25 ℃ with sunlight (L) and in the dark (D) for one month (*n*=3).

4. Conclusions

A green, simple, and effective extraction method using natural deep eutectic solvents (NADES) was established as well as an HPLC method to analyze the anthocyanin extracts. Under the optimized conditions, some NADES give the same yields as with acidified methanol. However, cyanidin is much more stable in some tested NADES than in acidified ethanol. NADES are alternative green solvents to harmful organic solvents for the extraction and storage of anthocyanins from plant material.

This study provides evidence on the extraction capacity, stabilization ability of NADES, and the compatibility of NADES extracts with reversed phase liquid chromatography. Based on these characteristics, NADES seem very promising for various applications in human health-related areas e.g. food, cosmetics and

pharmaceuticals. For designing application of NADES one should keep in mind their viscosity. For example, the viscosity instead of polarity plays an important role for NADES as extraction solvents. The high stability of cyanidin in NADES may be correlated with the viscosity via the establishment of hydrogen- bonds between cyanidin and the NADES molecules.

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