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Application of natural deep eutectic solvents to the extraction of anthocyanins from *Catharanthus roseus* with high extractability and stability replacing conventional organic solvents

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ABSTRACT

Natural deep eutectic solvents (NADES) have attracted a great deal of attention in recent times as promising green media. They are generally composed of neutral, acidic or basic compounds that form liquids of high viscosity when mixed in certain molar ratio. Despite their potential, viscosity and acid or basic nature of some ingredients may affect the extraction capacity and stabilizing ability of the target compounds. To investigate these effects, extraction with a series of NADES was employed for the analysis of anthocyanins in flower petals of *Catharanthus roseus* in combination with HPLC-DAD-based metabolic profiling. Along with the extraction yields of anthocyanins their stability in NADES was also studied. Multivariate data analysis indicates that the lactic acid–glucose (LGH), and 1,2-propanediol–choline chloride (PCH) NADES present a similar extraction power for anthocyanins as conventional organic solvents. Furthermore, among the NADES employed, LGH exhibits an at least three times higher stabilizing capacity for cyanidins than acidified ethanol, which facilitates their extraction and analysis process. Comparing NADES to the conventional organic solvents, in addition to their reduced environmental impact, they proved to provide higher stability for anthocyanins, and therefore have a great potential as possible alternatives to those organic solvents in health related areas such as food, pharmaceuticals and cosmetics.
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1. Introduction

A few years ago, our research group developed a new type of green solvent, natural deep eutectic solvents (NADES) [1,2]. These NADES are liquids made of primary metabolites (*e.g.,* sugars, sugar alcohols, organic acids, amino acids, and amines) bound together by strong intermolecular interactions, particularly hydrogen bonding. They have proved to present many advantages over the conventional solvents currently used for extraction [2], *e.g.,* negligible volatility, adjustable viscosity, and high solubilization strength. For example, some NADES have shown a very high solubilizing ability of rutin, in some cases as much as 12,000 times higher than water [1,2]. From an environmental and economic perspective, NADES also present great advantages concerning their bio-degradability, sustainability, low cost and simple preparation. All these properties indicate their great promise as extraction solvents for natural products [3] and their potential applications in health related areas such as food, pharmaceuticals and cosmetics.

In general, the components of NADES are characterized by the presence of several functional groups such as hydroxyls, carboxyl, or amino groups [1,2]. Those groups can form intermolecular hydrogen bond, leading to highly structured viscous liquids, which accounts for their specific physical properties and different solubilizing behavior compared to conventional solvents. Those liquids can also form hydrogen bonds with solutes thereby greatly increasing the solubility of compounds in NADES, *e.g.,* phenolic compounds. According to their components, NADES can be divided into five groups: ionic liquid types composed of an acid or a base, those with neutral compounds, sugar-based mixtures with an acid, sugar-based mixtures with a base and sugar-based NADES with an amino acid [2]. The different compositions of NADES result in a broad range of physical properties, probably leading to different behaviors in applications such as extraction, analysis and storage of natural products. In this study, anthocyanins were investigated for their extractability and stability as model compounds

Anthocyanins are a widespread group of water-soluble phenolic compounds that occur in plants. They exist mainly in flowers and fruits, being largely responsible for the orange, red, purple and blue

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colors that appear in plants [4]. Since the 1990s, there has been a renewed interest in anthocyanins because of their possible health benefits as antioxidant and anti-inflammatory agents, especially for the prevention of certain cardiovascular diseases and cancer [5–7]. Plant extracts containing anthocyanins, such as pomegranate extracts, have also been developed as "functional foods" and are included in "herbal/nutritional supplements" [8]. In plants, anthocyanins are mostly found as glycosides with sugar substituents and different aliphatic or aromatic carboxylic acids bound to the sugar units [7]. There are six major aglycones (anthocyanidins): delphinidin, cyanidin, peonidin, pelargonidin, petunidin and malvidin (Fig. S1), differing only in the number and position of hydroxyl or methoxyl substitutions in the B-ring [7].

The common solvents for the extraction of anthocyanins are mixtures of water with ethanol, methanol, or acetone [9,10]. The anthocyanin extracts or isolated anthocyanins are highly unstable being susceptible to degradation due to temperature, light, solvent and the solvent used for extraction. The instability of anthocyanins hampers their extraction, analysis and storage [4,11–13]. These factors make the protocol for sample preparation and analysis of anthocyanins complex, and the whole process can often be very time-consuming [14–16]. In general, it is recommended that the extraction of anthocyanins be performed in the dark and at a low temperature [17,18]. In view of all the clear shortcomings of the conventional methods, it is very necessary to develop a method using efficient new green solvents that could combine high extraction efficiency with increased stabilization of the anthocyanins.

Thus, in this study the extractability of anthocyanins by diverse NADES and their storage stabilization properties were explored. The simple extraction method was further evaluated by HPLC/DAD-based metabolomics of the resulting extracts. As a model, purple and orange petals of *Catharanthus roseus* were selected because they contain different kinds of anthocyanins. To study the stability of anthocyanins in NADES, the effects of temperature and storage time on NADES cyanidin solutions were evaluated. 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 *C. roseus* varieties, belonging to the Pacific series *i.e.,* Pacifica XP Apricot (orange color), and Pacifica Orchid Halo (purple color) were purchased from Intratuin, Pijnacker (Heerhugowaard, The Netherlands). Flower petals were collected, ground to powder with liquid nitrogen and freeze-dried in September 2009 during the blooming season of *C. roseus*. Analytical grade methanol and ethanol were purchased from Biosolve BV (Valkenswaard, The Netherlands). Malic acid, lactic acid, proline, glucose, fructose, sucrose, 1.2-propanediol, and choline chloride were purchased from Sigma (St. Louis, MO, USA) and the cyanidin from Carl Roth (Karlsruhe, Germany).

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) and glucose–fructose–sucrose (FGSH) were prepared following the method described in a previous report [2]. Proline aqueous solution (65 mg/ml) and malic acid aqueous solution (50 mg/ml) were prepared with deionized water.

2.3. Extraction methods optimization

2.3.1. Heating and stirring

Plant material (50 mg) was mixed with 1.5 ml NADES or 3% formic acid in methanol and stirred at 40 °C for 30 min in a sealed glass bottle. The mixture was then transferred to an Eppendorf tube, centrifuged at 1300 rpm for 20 min and filtered through a 0.45 μm cellulose acetate membrane filter. The filtrate was diluted 1:2 with 3% aqueous formic acid. All extractions were performed in triplicate. For *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 °C for 30 min. The extract was centrifuged, filtered, and diluted as described above.

2.4. Stability test

Solutions of 0.1 mg/ml cyanidin reference standard in LGH and 0.1 mg/ml in ethanol with 3% formic acid (0.1 mg/ml) were used for the following stability tests. For thermal stability, cyanidin solutions were transferred into vials with screw caps and placed in a preheated water bath at 80 °C, 60 °C and 40 °C, respectively. Three tubes of each temperature group were removed from the water bath at 10, 20, 40, 60, 80, 100, and 120 min, and rapidly cooled to room temperature and analyzed immediately. For the longer-term stability of the cyanidin solutions vials were stored at −20, 4 and 25 °C in the dark and samples of each group were analyzed after 1, 3, 7, 15, 30, 60, 90 days. All experiments were performed in triplicate.

2.5. Apparatus and analysis

HPLC analysis was carried out on an Agilent chromatographic system with a Phenomenex Luna C18 column (4.6 μm × 250 mm, 5 μm) at 35 °C. Samples were analyzed using gradient elution with 3% aqueous formic acid (A) and methanol with 3% formic acid (B) was 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 recorded at 520 nm. The injection volume was 10 μl. A UV–vis spectrophotometer (Shimadzu, Japan) was used for the spectrophotometric determination of the stability at a wavelength of 520 nm.

2.6. LC–MS analysis

Ultra performance liquid chromatography-time of flight-Mass spectrometry (UPLC-TOF-MS) analyses were performed on an UPLC-TOF-mass spectrometer Ultimate 3000 RS UPLC system (Thermoscientific, USA) coupled to a micro-TOF-QII mass spectrometer (Bruker Daltonics, Bremen, Germany) with an electrospray (ESI) interface and a diode array detector. The *C. roseus* extracts were analysed in positive mode. Nitrogen served as a curtain gas (10 psi). The *m/z* range was set to be 400–1000. For ESI the conditions were as follow: capillary voltage of 4000 V, source temperature of 250 °C, desolvation temperature of 250 °C, dry gas flow of 11.0 l/min and the nebulizer at 2.0 Bar. For positive ionization, all conditions were the same and the capillary voltage was 4000 V. Internal calibration was performed using a 10 mM sodium formate solution from Sigma–Aldrich (Steinheim, Germany). Samples of 5 μl were injected onto a Kinetex C18, 100 Å ~2.10 mm column, packed with 2.6 μm particles with a C18 UHPLC pre-column of SecurityGuard ULTRA Cartridges (Phenomenex, USA), eluted in gradient mode at a flow rate of 0.3 ml/min with the following solvent system: (A) 0.1% formic acid (v/v) in water; (B) 0.1% (v/v) formic acid in methanol. Analysis began with a gradient of 30–70% B in 18 min, followed by an isocratic step of 70% B for 1 min and a

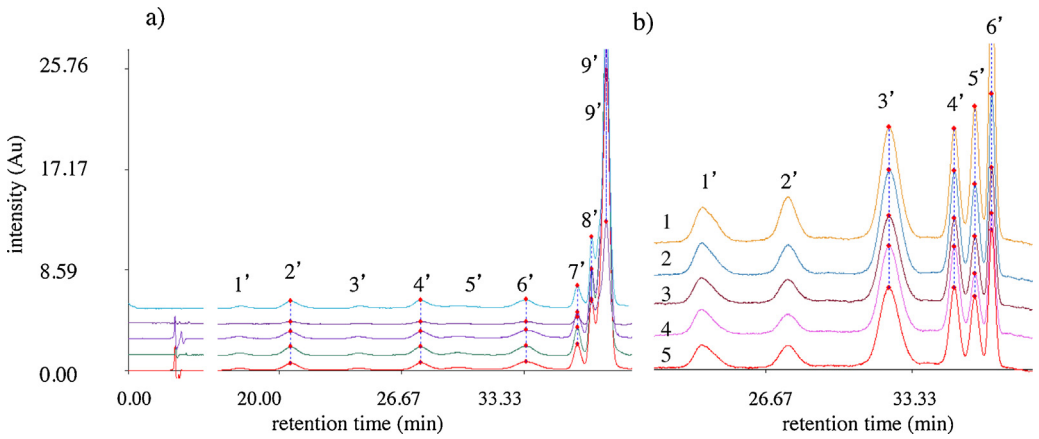

Fig. 1. HPLC-DAD chromatograms of anthocyanins from *Catharanthus roseus* with (a) purple and (b) orange flower petals extracted with different solvents at 520 nm. (From top to bottom (1) methanol; (2) lactic acid–glucose (LGH); (3) malic acid–choline chloride (MCH); (4) proline–malic acid (PMH); (5) glucose–choline chloride (GCH)). Identification of the HPLC peaks in (a): petunidin 3-*O*-(6-*O*-*p*-coumaroyl) glucose (7′); malvidin 3-*O*-(6-*O*-*p*-coumaroyl) glucose (8′); hirsutidin 3-*O*-(6-*O*-*p*-coumaroyl) glucose (9′). Identification of the HPLC peaks in (b):, 7-*O*-methylcyanidin (3′); 7,3′-*O*-dimethylcyanidin (5′).

re-equilibration of 1.5 min with 30% B. The total run time was 20.5 min. The temperature was maintained at 30 °C.

2.7. Data analysis

Calculation of kinetic parameters of cyanidin degradation at high temperature: the first-order reaction rate constants (k) and half-life ($t_{1/2}$), were calculated applying the following equation [20]:

$$\ln\left(\frac{c}{c_0}\right) = -kt$$

$$t_{1/2} = \frac{-\ln(0.5)}{k}$$

where $c/c_0 = A/A_0$, A_0 is the initial absorption of a cyanidin solution and A is the absorption value of the cyanidin solution after heating time (t) at the given temperature.

The integrated areas of peaks of anthocyanins in HPLC-DAD chromatograms from three replicates were analyzed with the SIMCA-P software (version 13, Umetrics, Umeå, Sweden) for principal component analysis (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 $\leq$0.05 considered as significant.

3. Results and discussion

3.1. Comparison of the extractability of anthocyanins

3.1.1. Comparison of the extractability of anthocyanins with NADES and methanol

C. roseus contains a number of anthocyanins that have been well studied in the past [21]. They have been found to contain high 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 low levels of petunidin 3-*O*-glucose along with trace amounts of some other anthocyanins [18].

C. roseus can have flowers of five different colors: pink, light pink, purple, red and orange and their anthocyanin profile differs for each color. Chromatograms of petals of the purple and orange flowers exhibit more peaks at 520 nm than those of the other colors, as shown in a previous study (Fig. S2). The purple- and orange-colored petals of *C. roseus* were thus selected to investigate the extractability of anthocyanins with NADES.

The HPLC chromatograms at 520 nm of some of the tested NADES and 3% methanolic formic acid extracts showed the same qualitative profiles of anthocyanins. Methanol with 3% formic acid was selected as the reference because of its high extraction yield of anthocyanins [17,22]. Nine peaks were observed for the purple petal extracts (Fig. 1a) and 6 for the orange petal extracts (Fig. 1b). 3% methanolic formic acid extracts of purple and orange petals were further investigated for major anthocyanis by UPLC-MS. In the extracts of purple *C. roseus* petals petunidin 3-*O*-(6-*O*-*p*-coumaroyl) glucose ([M$^+$] 625, peak 7′), malvidin 3-*O*-(6-*O*-*p*-coumaroyl) glucose ([M$^+$] 639, peak 8′), hirsutidin 3-*O*-(6-*O*-*p*-coumaroyl) glucose ([M$^+$] 653, peak 9′) could be identified by UPLC-MS [18]. In the extract of orange *C. roseus* petals, two anthocyanins with molecular ions [M]$^+$ at m/z 609 composed of 7-*O*-methylcyanidin and [M]$^+$ (peak 3′) at m/z 623 composed of 7,3′-*O*-dimethylcyanidin (rosinidin) (peak 5′) and were identified and detected at 520 nm which were previously reported in *C. roseus* [19]. [M]$^+$ of peak 2′ was at m/z 579 and of peak 4′ at m/z 593 in the orange petals extract of probably other unknown 3-*O*-glycosides.

To quantify the different extractability of NADES and methanol with 3% formic acid for anthocyanins, principal component analysis (PCA) was applied with the peak areas of all peaks in chromatograms at 520 nm as variables. The score plot of PCA (Fig. 2a) with the first two components (R^2 = 0.94 and Q^2 = 0.67) shows a separation of the purple petal extracts into 2 groups in 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 area of PC1 (group II). The score plot indicates that LGH, PCH and 75% FGSH have similar characteristics to acidified methanol in extracting anthocyanins from purple *C. roseus* flowers. The loading plot (Fig. 2b) shows that all peaks are located in the positive area of PC1 (group I), indicating that solvents in the group I (PCH, LGH, 75% FGSH and acidified methanol) have a higher extraction capacity than those in group II (PMH, MCH and GCH). It allowed us thus to conclude that LGH, PCH and 75% FGSH are suitable extraction solvents for these anthocyanins, being as efficient as acidified methanol.

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 all the extracts to be separated into two groups by PC1 (Fig. S3a), with acidified methanol, LGH and PCH in the positive area of PC1 (group I) and PMH, MCH, and GCH in the negative area of PC1 (group II).

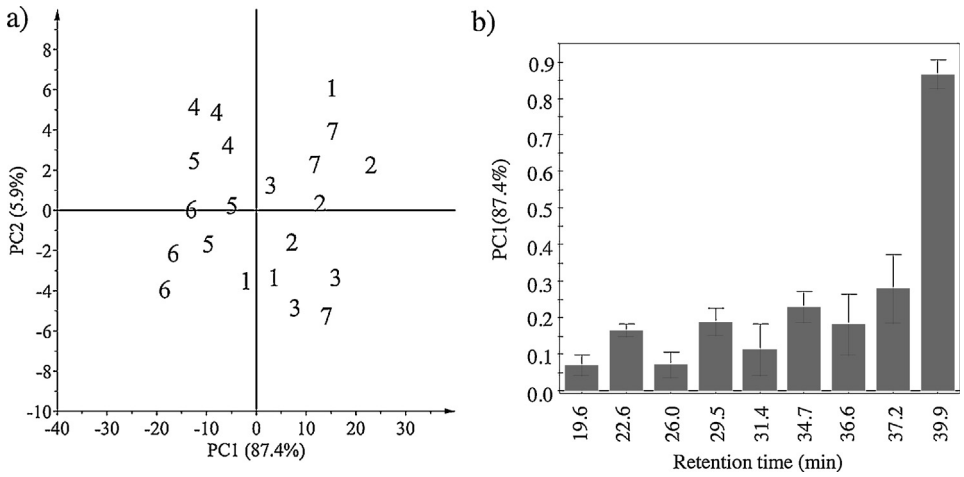

Fig. 2. Score plot (a) and loading plot (b) of principal component analysis of extracts of *Catharanthus roseus* purple flowers obtained with different NADES and methanol ((1) methanol 3% formic acid; (2) 1,2-propanediol–choline chloride–water; (3) lactic acid–glucose–water; (4) proline–malic acid; (5) malic acid–choline chloride; (6) glucose–choline chloride; (7) 75% (v/v) glucose–fructose–sucrose).

The loading plot (Fig. S3b) shows that all peaks of anthocyanins are located in the positive area of PC1, indicating that group I (LGH, PCH and acidified methanol) solvents give a higher extraction yield than solvents in group II.

A further ANOVA test on the average peak area of anthocyanins in HPLC-UV chromatogram confirmed that LGH, PCH and 75% FGSH have the same extraction yields of anthocyanins as acidified methanol, and are the most efficient of all tested NADES. The ANOVA showed that the area of the peak (rt = 39.9 min) was significantly higher in LGH, PCH, 75% FGSH, and acidified methanol than in the other NADES (Fig. S4), which confirmed the results from PCA that show that the solvent strength of LGH, PCH, and 75% FGSH is similar to acidified methanol and much higher than the other NADES tested.

3.1.2. Extractability of anthocyanins and the physicochemical behavior of NADES

The high extraction yield of PCH, LGH and 75% FGSH may be related to their physicochemical properties such as their viscosity, polarity and their chemical composition or pH. To test the influence of these features, the following 6 typical NADES with different physical properties were selected for extraction: PCH, LGH, PMH, MCH, GCH, and FGSH [2]. These six typical NADES possess different viscosity from 35.7 cP to 548.6 cP (Table S1), polarity in the form of E_{NR} from 44 kcal/mol to 50 kcal/mol and pH from 3 to 7, which has been reported in our previous papers [2,23].

Our results showed a clear inverse correlation of the extraction yield with viscosity, LGH and PCH being the most efficient and least viscosity (*ca.* 40 cP) among all tested NADES. All others had a much higher viscosity: GCH (479.6 cP), PMH (330.9 cP), MCH (548.6 cP) [2]. In the case of FGSH, it was diluted with 25% water (v/v) for use, resulting in a similar viscosity as water. This was not the case for polarity where a relationship between the polarity of the NADES and the extractability of the anthocyanins was no found. In the case of conventional solvents, the extraction efficiency can be predicted by their polarity. Among the tested NADES, LGH and MCH are the most polar (44 kcal/mol), followed by PMH (48 kcal/mol), sugar/sugar alcohol–choline (49 kcal/mol) and finally PCH (50 kcal/mol) [2], proving that there is no clear correspondence with their extraction capacity. Similarly, it was not

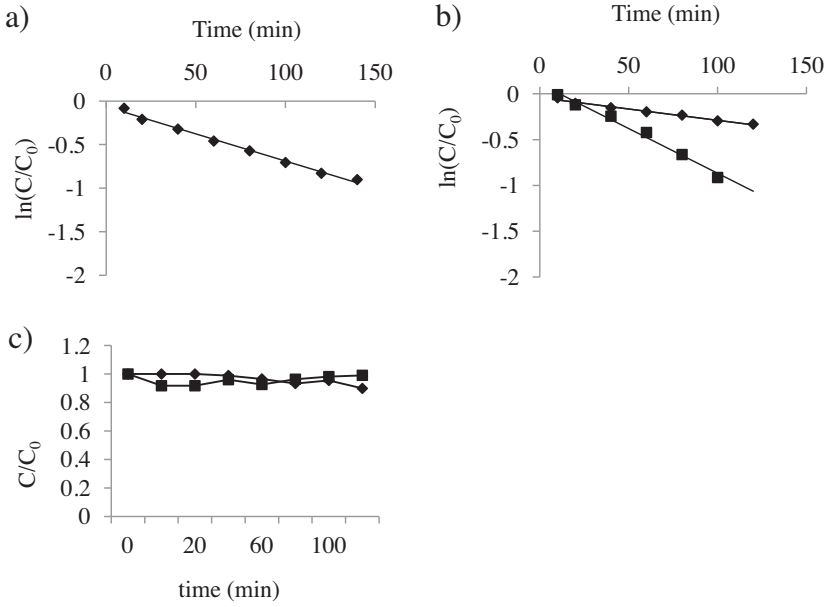

Fig. 3. Stability of cyanidin in lactic acid–glucose (LGH ♦) and ethanol with 3% formic acid (■) at (a) 80 °C, (b) 60 °C and (c) 40 °C in the dark.

Table 1
Influence of three different extraction methods on the relative extraction yield of anthocyanins from *Catharanthus roseus* with purple flowers ($n = 3$).

Peak no.	Retention time (min)	Peak area ratio[a]		
		Sonication (25 °C)	Sonication (40 °C)	Sitrring (40 °C)
1	19.6	0.6	0.8	1.0
2	22.6	0.5	0.6	1.0
3	26.0	0.6	0.4	1.0
4	29.5	0.5	0.5	1.0
5	31.4	0.4	0.3	1.0
6	34.7	0.6	0.6	1.0
7	36.6	0.5	0.7	1.0
8	37.2	0.5	0.7	1.0
9	39.9	0.5	0.7	1.0

[a] The relative extraction yield is expressed in peak area ratio of the peak in the chromatogram of the extract as compared to the control (40 °C, stirring) as reference.

possible to find any correlation with the acid or basic nature of the NADES components and their extraction yields. This is different to what can be observed with the conventional extraction methods for anthocyanins that include a low amount of acid to the organic solvent to achieve better yields [17]. The pH of aqueous NADES (diluted with 90% (v/v) of water) differ according to the presence of acids or bases [23] and LGH and PCH which have the same extractability have different pH values. On the other hand, 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 correlated to the viscosity of NADES rather than their polarity and pH.

3.2. Optimizing extraction and analysis method for anthocyanins with NADES

3.2.1. Extraction methods optimization

Three extraction methods—mechanical stirring at 40 °C, ultrasonication at 40 °C and 25 °C were examined for the extraction of anthocyanins from flower petals of *C. roseus*. Among these, the least efficient was ultrasonication at 25 °C but increasing the temperature to 40 °C produced a 2–20% improvement in the yield. The best extraction yield was obtained by stirring at 40 °C and it was 35–55% greater than that obtained with sonication at 25 °C (Table 1). The effect of extraction time on the yield obtained with the stirring method was evaluated by comparing the profile of extracts obtained throughout a range of 30–90 min extractions. The results showed there was no difference in extraction yields with 30 and 60-min extraction time but at 90 min the areas of all peaks decreased (Table S2). It might be due to the fact that while a prolonged extraction time facilitates the dissolution of compounds it may also lead to the formation of artifacts due to the degradation of compounds. Thus, the optimum conditions, *i.e.*, stirring at 40 °C for 30 min was selected for this study.

Comparing with conventional solvents, a disadvantage of NADES is their high viscosity, which causes slow mass transfer and results in a decrease in their extraction ability. In order to improve this, the above three approaches were explored with the purpose of decreasing the viscosity and improving extraction efficiency. In a previous study, we showed that while increasing the temperature leads to a decrease in viscosity of NADES [2], it also decreases the stability of anthocyanins that are thermally unstable [20]. Thus, a mild temperature of 40 °C was selected. Another way to increase mass transfer and to speed up the diffusion of compounds in NADES is to apply external forces such as stirring and sonication. Stirring is the simplest way to speed up the diffusion rate of the compounds in the liquid. Ultrasonication combined with mild heating might also 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 [24]. 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 has been widely used in the extraction of metabolites from plant materials [25,26]. However, with NADES as extraction solvents, sonication proved to be less efficient than stirring due to their high viscosity.

3.2.2. The compatibility of NADES with reverse phase HPLC

Because the solvent features of NADES are so different from conventional ones, it was necessary to study the chromatographic behaviors of their components. For this aqueous solutions of malic acid and proline individually and their deep eutectic mixture (PMH) were injected separately and their retention times were found not to overlap with target anthocyanins. The chromatographic behavior of malic acid in different dilutions of NADES (PMH and MCH) in water was compared with that of an aqueous solution of malic acid (Table S3). The retention time and peak shapes of malic acid in the different dilution of NADES were also similar to those obtained with 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 progressive water dilution and even breaks completely above 50% (v/v) water [2,27]. The chromatographic behavior of anthocyanin extracts in methanol and different NADES were compared. The profiles of all extracts were qualitatively similar (considering the retention time and peak shape) (Fig. 1), indicating that components of NADES have little effect on the chromatographic profiles of anthocyanin extracts used in this study. In conclusion, in this case, it is recommended to dilute NADES extracts to double their volume with water before their analysis.

Regarding the chromatographic system, it is also recommendable to start with a low percentage of organic solvent in the initial composition of the mobile phase as this may affect the peak shape of solutes. Starting with a high amount of organic solvent will result in poor peak shapes of analytes. To ensure a higher resolution in the chromatographic profiles of analytes, it is thus better to set the ratio of the starting mobile phase with a high percentage of water. Additionally, a high ratio of water will dilute the NADES and will not cause an unwanted retention of its ingredients in the columns. This is due to the inherently low retention of these hydrophilic ingredients on the reversed-phase stationary phase.

3.3. Stability of anthocyanins in NADES

As part of the validation of any extraction method it is very important to consider the stability of target solutes during extraction and batch analysis, as well as during sample storage. Therefore, the effects of solvent, temperature and storage time on the stability of anthocyanins in NADES were investigated using cyanidin in LGH as a marker. To avoid the toxicity and volatility of methanol, acidified ethanol was used as a solvent for the stability tests. Fig. 3a–c shows the degradation curve of cyanidin at high temperature (40–80 °C) in LGH and ethanol with 3% formic acid. The results showed that cyanidin was more stable in LGH than in acidified ethanol at 60 °C and stable in both solvents at 40 °C for 1.5 h in the dark. The degradation of anthocyanins over time follows a first-order reaction at 60 °C and 80 °C, which is in agreement with a previous report on the degradation of anthocyanins [20]. The half-life time ($t_{1/2}$) of cyanidin was more than 3 times greater in LGH than in acidified ethanol at 60 °C (Table 2) and even greater

Table 2
Degradation kinetic parameters of cyanidin in lactic acid–glucose (LGH) and ethanol with 3% formic acid at 60 and 80 °C including reaction rate constants (k) and half-lives ($t_{1/2}$), and the degradation functions ($n = 3$).

Degradation kinetics parameters of cyanidin					
Temperature (°C)	Solvent	k	R^2	$t_{1/2}$ (min)	Function
80	LGH	0.0062	0.9921	111.8	$y = -0.0062x - 0.0653$
60	LGH	0.0025	0.9844	277.26	$y = -0.0025x - 0.041$
60	EtOH	0.0096	0.9389	72.2	$y = -0.0096x + 0.1241$

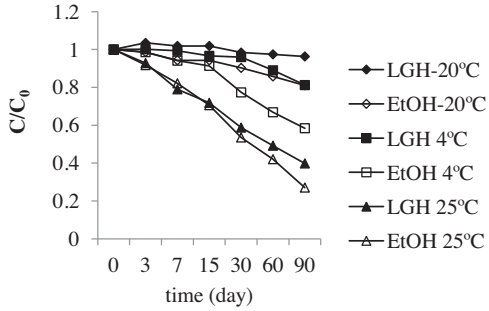

Fig. 4. Stability during storage of cyanidin in lactic acid–glucose (LGH) and ethanol with 3% formic acid at (a) −20 °C, 4 °C and 25 °C in the dark for 3 months.

at 80 °C in LGH than at 60 °C in acidified ethanol. Therefore, cyanidin is much more stable in LGH than in acidified ethanol at high temperature in the dark.

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. 4). The different degradation curves show that cyanidin is 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 and acidified ethanol at −20 °C. Cyanidin solutions maintained the same absorbance at 520 nm in LGH at −20 °C for 3 months, at 4 °C 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, the 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 °C in the dark up to 3 months.

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 have intermolecular interactions, mainly hydrogen bonding with the carboxyl and hydroxyl groups of cyanidin, as was observed for quercetin in xylitol–choline chloride [2]. This interaction decreases the movement of solute molecules, reducing its contact time with oxygen at the interface of NADES and air, and consequently reducing oxidative degradation, the major degradation mechanism. This hypothesis is supported by other findings such as the greater stability of cyanidin in a gel model system with pectin than in presence of an aluminum ion, which was due to the anthocyanin–metal interaction [28]. Similarly, manually squeezed strawberry and elderberry 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 [29]. Thus, the increased interaction with other molecules improves the color stability of anthocyanin.

4. Conclusion

A green, simple, and effective extraction method using natural deep eutectic solvents (NADES) including an HPLC method to analyze the obtained anthocyanin extracts was developed. Under the optimized conditions, some NADES give similar yields to those obtained with acidified methanol, the conventional solvent. Moreover, cyanidin is much more stable in some tested NADES than in acidified ethanol. Thus, NADES represent an efficient green alternative to harmful organic solvents for the extraction and storage of anthocyanins from plant material.

This study provides evidence on the extraction capacity and stabilization ability of NADES, as well as the compatibility of NADES extracts with reversed-phase liquid chromatography analysis. Based on these characteristics, the NADES can be proposed for very promising applications in human health-related areas *e.g.,* food, cosmetics and pharmaceuticals. When designing NADES applications it is important to keep their viscosity in mind. For example, it is the viscosity rather than their polarity that plays an important role in the efficiency of NADES as extraction solvents. The stability of cyanidin in NADES proved to be remarkably higher than in organic solvents and may be due to the establishment of extensive hydrogen bonding between cyanidin and NADES components.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.01.037.

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