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

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Chapter 5

Natural Deep Eutectic Solvents providing enhanced stability of natural colourants from safflower (*Carthamus tinctorius*)

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Abstract

As promising new solvents for food, cosmetic and pharmaceutical industry, natural deep eutectic solvents (NADES) show a good solubilizing capacity for compounds with diverse polarities. In addition to the solubility data, the stability of compounds in NADES should be investigated for further applications. As a model, the stability of carthamin, an unstable pigment, was evaluated in some typical NADES. The different factors affecting the stability of compounds are heat, light, and storage time. In all tested conditions, carthamin was more stable in sugar-based NADES than in water or 40% ethanol. Moreover, carthamin together with hydroxysafflor yellow A and cartormin, the main active compounds and major pigments in safflower, exhibited improved stability in sugar-based NADES extracts at ambient conditions in daylight. Notably, NADES extracts with low water content and high viscosity exhibited even better stability. The strong stabilization capacity of some NADES was found to be due to the formation of hydrogen bonding interactions between solutes and NADES molecules, which is related with the high viscosity of NADES. These results show the stabilizing ability of NADES for phenolic compounds and holds great promise for their applications in food, cosmetic and pharmaceutical industry.

Key words: natural deep eutectic solvents; stabilizing capacity; carthamin; safflower; water content; hydrogen bonding.

1. Introduction

To extend the range of green solvents, we proposed natural ionic liquids and deep eutectic solvents (NADES) for applications in health-related areas such as food, pharmaceuticals and cosmetics (Choi *et al*., 2011; Dai *et al*., 2013). NADES are liquid supermolecules made of natural primary metabolites bound together by inter-molecular interactions, particularly hydrogen bonding. They have several advantages over synthetic ionic liquids, e.g. their low cost, biodegradability, non-toxicity, sustainability, and simple preparation methods. Moreover, they show very good physicochemical properties as solvents: negligible volatility, liquid state even below 0 ^{o}C, adjustable viscosity, wide polarity range, and high solubilization strength for a wide variety of compounds (Dai *et al*., 2013). All those properties imply their potential for various types of applications in health related areas. Until now, they have been used in metabolite extraction (Usuki *et al*., 2011), enzyme stability and enzymatic reactions (Kragl *et al*., 2002; Kaar *et al*., 2003; Gorke *et al*., 2008). Few studies have been done to evaluate the stabilization ability of NADES for natural products. Undoubtedly, it is essential to determine the stability of compounds in NADES in order to evaluate its applicability to all kinds of natural products extraction and processing.

Natural deep eutectic solvents (NADES) have a great potential as stabilizing media for e metabolites due to their unique physicochemical properties. In our view, NADES occur in all organisms and cells, where NADES exist around the membranes and are involved in the biosynthesis, solubilization and storage of various poorly water-soluble metabolites and unstable compounds in cells (Choi *et al*., 2011; Dai *et al*., 2013). This hypothesis does give a number of ideas for applications of NADES. For example, carthamin is stable in the plant which implies that in safflower NADES may stabilize carthamin as well as other pigments.

Safflower (Flos carthami), the corolla from *Carthamus tinctorius* L. (Asteraceae) is used as a natural dye, food additive, and cosmetic. Also, it is widely used as traditional medicine for cardiovascular diseases (Huang *et al*., 1987; Shi and Liu, 2006; Zhou *et al*., 2008). Antioxidant and neuroprotective properties have also been reported for safflower extracts (Hiramatsu *et al*., 2009; Lee *et al*., 2009). The dried petals of safflower contain yellow and red pigments. Safflower yellow pigment is one of the few water-soluble yellow pigments found in nature. The major components of safflower yellow pigments are hydroxysafflor yellow A (HSYA), safflor yellow B and some other minor components, such as cartormin (Kazuma *et al*., 2000; Yin and He 2000). HSYA was reported to have an antithrombotic and neuroprotective effect (Zhu *et al*., 2003; Wei *et al*., 2005). Carthamin is the major red pigment in safflower and it has antioxidant activity (Takahashi *et al*., 1982; Wang and Zheng 2006).

Thebasic structure of those pigments is a *C*-glucosyl quinochalcone (Jin *et al*., 2008; Kazuma *et al*., 2000).

However, carthamin is very unstable in an aqueous solution. It is usually extracted with an alkaline solution, but the red colour fades progressively to reddish orange, orange-yellow, yellow and light yellow (Saito *et al*., 1992; Wang and Zheng, 2006). Carthamin has been reported to be more stable in alkaline than in acid or neutral conditions (Fatahi *et al*., 2009). But it degrades fast when heating, having a half-live in alkaline conditions of 12.5 hours at 25 ^{o}C and 0.75 hours at 60 ^{o}C (Kim and Paik, 1997). The presence of NADES in the plant may, to some extent, solve the instability of carthamin in the plant cells.

NADES have totally different characteristics if compared to conventional solvents. The major components of NADES are natural primary metabolites, e.g. sugars, sugar alcohols, organic acids, amino acids and amines, which have several hydroxyl groups, carboxyl groups, or amino groups (Choi *et al*., 2011; Dai *et al*., 2013.) Hydrogen-bonding interactions appear between molecules with these groups, leading to highly structured viscous liquids. Those liquids can in turn, form hydrogen bonds with solutes (Dai *et al*., 2013) increasing their solubility, e.g. phenolic compounds. NADES are like liquid crystals in which all molecules are arranged in a matrix with optimum interactions via inter- and intra- molecular H-bonding of the constituents. Studies are needed to explore the full potential of NADES as solvents for food, medicine and cosmetics.

This chapter describes the stabilizing ability of some typical NADES for carthamin. The stabilizing mechanism was investigated, and NADES with high stabilization ability for unstable phenolic compounds like carthamin were developed. As an important factor, the effect of the water content in NADES on the stability of carthamin was investigated.

2. Materials and methods

2.1. Chemicals, material and reagents

Carthamin was isolated from safflower bought from Xinjiang province in China. The plant material was identified by Dr. Young Hae Choi, and a voucher specimen (NPL-safflower-0913) was deposited in the Natural Products Laboratory, Institute of Biology, Leiden University. The dry plant material was ground into a powder in a blender with liquid nitrogen. Ethanol of analytical grade and acetonitrile of HPLC grade were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was of deionized water quality. Malic acid, lactic acid, proline, sucrose, glucose, xylitol and choline chloride were purchased from Sigma (St. Louis, MO, USA).

2.2. Solvent and sample preparation

All NADES including glucose-choline chloride (GCH); sucrose-choline chloride (SuCH); proline-malic acid (PMH); lactic acid-glucose (LGH); xylitol-choline chloride (XoCH) were prepared using mild heating combined with stirring at 50 ^{o}C (Dai *et al*., 2013). The 40% EtOH, 90% GCH, 75%, 50% and 25% PMH and SuCH were prepared diluting a certain volume of NADES with deionized water.

Carthamin solutions were prepared by dissolving carthamin in each solvent (water, 40% EtOH, NADES) with agitation for 30 min at room temperature. The samples were transferred into an Eppendorf tube, centrifuged at 1300 rpm for 20 min and then the supernatant was used for further tests. Extraction was performed in sealed bottles with 50 mg plant material and 3 mL NADES or 40% ethanol, heating and stirring at 40 ^{o}C for 30 min. The sample was transferred into an Eppendorf tube, centrifuged at 1300 rpm for 20 min and then the supernatant was filtered through a 0.45 μm cellulose membrane. The resulting solutions were used to test the effect of storage at ambient room conditions with daylight on the stability of dissolved phenolic compounds.

2.3. Stability tests

The effects of heating, light, ambient conditions in daylight, storage time, and water content in NADES on the stability of carthamin and safflower extract were investigated with the methods described bellow.

For thermal stability, carthamin solutions were put in glass vials with screw-caps and placed in a preheated water bath at 80 ^{o}C, 60 ^{o}C and 40 ^{o}C. Three tubes of each group were removed from the water bath after 10, 20, 40, 60, 80, 100, 120 min and rapidly cooled to room temperature.

The effect of light from artificial light was determined with carthamin solutions at room temperature. Tubes with the test solutions were placed one meter below a lamp (TL 40 W, Philips) or covered with aluminum foil, and three tubes of each group were taken at day 0, 3, 7, and 15 for UV spectroscopy.

The effect of storage stability was investigated at -20 ^{o}C and 4 ^{o}C in the dark with carthamin solution and three tubes of each group were tested at day 0, 3, 7, 15, 30 and60.

The effect of ambient conditions in daylight was studied with carthamin solutions and safflower extract solutions. Each solution was exposed to room conditions in the daylight of late spring in The Netherlands and three samples of each group were removed at day 0, 3, 7, 15 for HPLC analysis.

The effect of water content in NADES on the stability of carthamin was investigated with two NADES (PMH and SuCH) at 4 ^{o}C and -20 ^{o}C in the dark. PMH and SuCH were used with 0%, 25%, 50%, and 75% (v/v) water. All stability tests were done by triplicate.

2.4 Apparatus and analysis

UV-Vis spectrophotometer (Shimadzu, Japan) was used for the stability test of carthamin using a wavelength of 520 nm. Extracts were analyzed with a

Agilent 1200 HPLC-DAD on a Phenomenex Luna C18(2) (4.6 μm x 250 mm, 5 μm) column. The mobile phase consisted of 0.5% H_3PO_4 (A) and acetonitrile (B) in a linear gradient program as follows: 5%-11% B (0-10 min), 11%-14% B (10-16 min), 14% B (16-23 min), 14%-20% B (23-30 min), 20%-35% B (30-70 min), 35%-60% B (70-80 min) at the flow rate of 1.0 mL/min (Wang *et al*., 2008). The injection volume was 10 μL. Chromatograms were recorded at 520 nm, 403 nm, and 280 nm. FT-IR spectra over the range from 4000 to 300 cm^{-1} were registered at room temperature (25 ^oC) using a Bruker FT-IR spectrometer. The pH of the diluted NADES with 90% (v/v) deionized water was tested with pH indicator paper (Merck, Darmstadt, Germany).

2.5 Data analysis

Calculation of kinetic parameters of carthamin degradation at high temperature: the first-order reaction rate constants (k) and half-lives ($t_{1/2}$), for degradation of 50% of carthamin, were calculated by the following equations (Kırca *et al*., 2007):

$$\ln(C/C_0) = -k\,t$$

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

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.

3. Results and discussion

To test the effect of viscosity, polarity, and composition of NADES (acidic or basic) on their stabilization ability, the following five typical NADES were selected: PMH, LGH, GCH, SuCH and XoCH (Table 1). Physical properties of NADES with diverse compositions show that all the NADES are different in composition (acidic or basic), viscosity and polarity (Dai *et al*., 2013). LGH and PMH are acidic with a pH=3 when diluted with 90% (v/v) of water, and the other NADES have pH values from 6 to 7 after dilution (table 1). Regarding their viscosity, PMH, SuCH and GCH are the most viscous, followed by XoCH, while LGH has the lowest viscosity. LGH is the most polar of the studied NADES, whereas PMH is similar to water, and sugar/sugar alcohol-choline is slightly less polar than water. It was reported that 40% ethanol has the highest extraction ability for the pigments from safflower (Zhang *et al*., 2009) while water is a common general extraction solvent for the safflower yellow pigment (Wang and Zhang, 2007; Zhu and Pan, 2007). Thus, the stabilization ability of NADES was investigated using both water and 40% ethanol as references.

3.1 Stability of carthamin during heating

Table 1. Some physical properties data of the selected natural deep eutectic solvents (NADES) and a summary of the stability of typical phenolic compounds (carthamin (1), hydroxysafflor yellow A (2), and cartormin (3)) in NADES with water and 40% ethanol as references.

	physical data			**conditions**[c]						
solvents	**compositions (molar ratio)**	**viscosity**[a] **(40 °C)**	**pH**[b]	**heating (40-80 °C)**	**light from lamp (~25 °C)**	**storage time**		**sunlight at ambient conditions (~25 °C)**		
						4 °C	**-20 °C**			
				1				**1**	**2**	**3**
LGH	lactic acid:glucose (5:1)	37	3		-	+	++	-	-	-
PMH	proline:malic acid (1:1)	251	3		-	-	++	-	-	-
GCH	glucose:choline chloride (2:5)	397	6-7		++	++	++	++	++	++
SuCH	sucrose:choline chloride (1:4)	581	6-7		+	++	++	+	++	++
XoCH	xylitol: choline chloride (1:4)	86	6-7	+						
water		1	7	-	-	-	-	-	+	+
EtOH (40%)	40 v% ethanol in water				-	-	-	-	+	+

[a] the viscosity and polarity data are for the pure NADES from previous report (Dai, *et al*., 2013).
[b] the pH value were detected with 90% (v/v) water dilution of NADES.
[c] "-" not stable; "+" more stable than "-" ; "++" more stable than"+"

The degradation rate of carthamin in aqueous solution was reported to increase at high temperatures by Fatahi *et al*. (2009). This was also observed in our experiments, in which the degradation rate of carthamin increased with increasing temperature (Fig. 1). However, carthamin was much more stable in XoCH than in water. At all tested temperatures, the degradation rate of carthamin in XoCH was much slower than in water. At 60 and 40 °C, first-order kinetics were observed for the degradation of carthamin in both XoCH and water, which is in agreement with a previous report on the degradation of carthamin in aqueous solution (Kim and Paik, 1997). At 60 °C, the half-live ($t_{1/2}$) of carthamin in XoCH was more than twice that in water (Table 2). Furthermore, if compared to the $t_{1/2}$ of carthamin at 60 °C, the $t_{1/2}$ in XoCH is 5 times higher and 8-time higher than that in XoCH and water at 40 °C, respectively. Therefore, compared with water, XoCH has a clear protective effect on carthamin against thermodegradation.

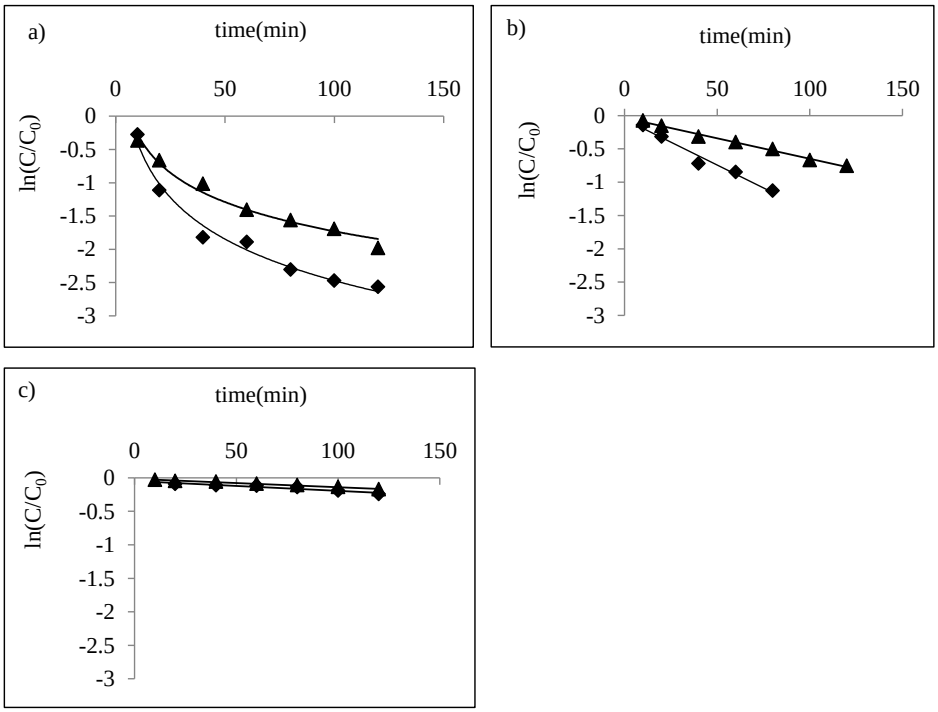

Fig.1. Stability of carthamin in xylitol-choline chloride (▲) and water (♦) at high temperature (**a**) 80 °C, **b**) 60 °C and **c**) 40 °C) (n=3). C_0 is the initial concentration of diluted carthamin and C is the concentration value of diluted carthamin after heating time (t) at a given temperature.

3.2 Stability of carthamin in light

The stability of carthamin in light was investigated at room temperature exposing a solution to 24 hours light under a daylight lamp over a 15-day period.

The reference was a similar solution that was kept in the dark. The effect of light on the stability of carthamin differed according to the solvents (Fig. 2a). The degradation curve of carthamin in light and in the dark overlapped in three of the solvents, GCH, SuCH and water, implying that light has no obvious effect on the stability of carthamin in those three solvents at room temperature for 15 days (Fig. 2b). With light, carthamin degraded faster than in the dark when dissolved in LGH, and 40% ethanol, but especially more in PMH (Fig. 2c), suggesting that light accelerates the degradation of carthamin in these solvents at room temperature.

Table 2. Degradation kinetics parameters of carthamin in water and xylitol-choline chloride (XoCH) at high temperature including reaction rate constants (k) and half-lives ($t_{1/2}$), and the degradation functions (n=3).

temperature	solvent	k	R^2	$t_{1/2}$	function
80 °C	XoCH	0.0482	0.9757		y = -0,6345Ln(x) + 1,1927
	H_2O	0.9012	0.9817		y = -0,9012Ln(x) + 1,6792
60 °C	XoCH	0.0061	0.992	113.6	y = -0,0061x - 0,0348
	H_2O	0.0138	0.9723	50.2	y = -0,0138x - 0,0546
40 °C	XoCH	0.0012	0.9841	577.6	y = -0,0012x - 0,0155
	H_2O	0.0017	0.9532	407.7	y = -0,0017x - 0,756

As regards different solvents, comparing the degradation curve of light-exposed solutions of carthamin in 90% GCH and 75% SuCH, the stability was very much higher than 40% ethanol and water solution. Thus, SuCH and GCH are good solvents for carthamin that exert a higher stabilization effect on solutes than conventional solvents and show no obvious effect from light.

3.3 Stability of carthamin according to storage time

At -20 °C, carthamin was stable in all tested solvents over a 7- day period. After 15 days, it was still stable in SuCH, and less than 10% degradation occurred in other NADES (PMH, 90% GCH, LGH), while substantial degradation was observed in water and 40% ethanol (Fig. 3a). At 4 °C, carthamin remained stable in SuCH over one month, while it showed degradation in other solvents including NADES. The stability of carthamin solutions decreases in the following sequence: SuCH> GCH> LGH> PMH=40% EtOH> water (Fig. 3b). In particular, at 4 °C the degradation of carthamin was below 5% in the NADES in the first 3 days, while it was near 15% in ethanol and 38% in water. Comparing -20 °C and 4 °C, the degradation of carthamin was increased from 35% to 61% in ethanol and from 16% to 26% in GCH after 30 days. Thus, NADES also exert a stabilizing effect on carthamin during storage. Therefore, carthamin can be preserved in SuCH at 4 °C for at least one month.

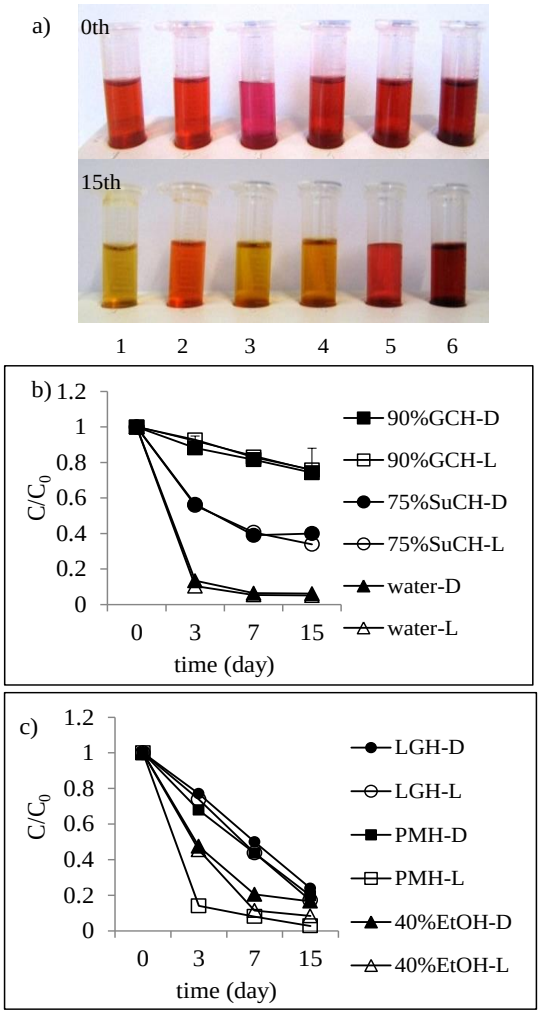

Fig. 2. Stability of carthamin in light from lamp at ambient temperature **a**) Pictures of carthamin solution before and after 15 days' 24-hour light exposure (**1**, water; **2**, 40% EtOH; **3**, lactic acid-glucose (LGH); **4**, proline-malic acid (PMH); **5**, sucrose-choline chloride (SuCH); **6**, 90% (v/v) glucose-choline chloride (GCH)) and degradation curve of carthamin in two groups of solvents with daylight (L) and in the dark (D) (group **b**: 90% GCH, 75%SuCH, and water; group **c**: LGH, PMH, and 40% (v/v) ethanol) (*n*=3).

Carthamin is more stable in SuCH and GCH than in PMH and LGH in all forementioned conditions (table 1). The big difference among those NADES is their viscosity; SuCH and GCH have a much higher viscosity than PMH and LGH. Thus, the stabilization ability of NADES may have a relationship with their viscosity. Moreover, the viscosity of NADES is affected by temperature

inversely, so that the viscosity of NADES decreases with increased temperature, which may partly explain why the stability of carthamin decreases with increased temperatures. For instance, in the case of PMH and LGH, the stability of carthamin decreased significantly when the temperature increased from -20 ^{o}C to 4 ^{o}C. The high viscosity at low temperature decreases the movement of molecules, allows stable molecular interactions between solvents and carthamin (section 3.6), and therefore possibly reduces the degradation of carthamin.

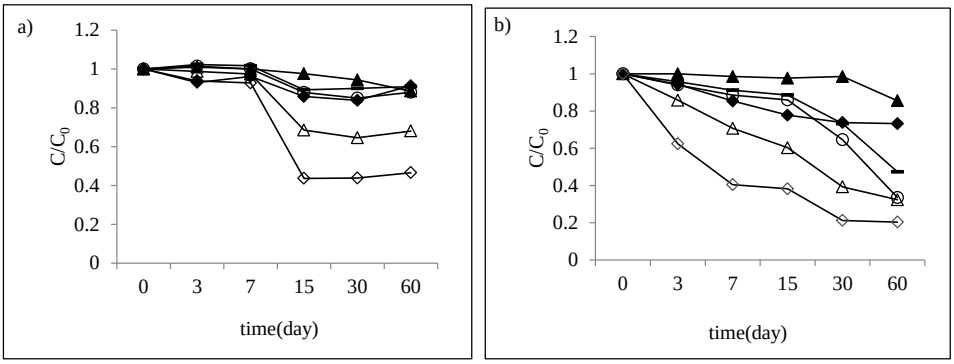

Fig. 3. Stability of carthamin standard in different natural deep eutectic solvents in two months at **a**) -20 ℃ and **b**) 4 ℃ (SuCH (▲); 90% GCH (♦); LGH (-); PMH (○);40% EtOH (Δ); water (◊))(*n*=3).

3.4 Stability of carthamin and safflower extraction at ambient conditions in sunlight

The red colored safflower was selected to explore the stabilizing ability of NADES for aromatic pigments with different polarities at ambient conditions in sunlight. The retention time in HPLC profile corresponded well with their polarity. There are numerous peaks in the UV-trace, including those visible at different wavelengths (280 nm, 520 nm and 403 nm) (Fig. 4). Because of the complexity derived from overlapping and minor compounds, three typical peaks with different retention times (Tr) were selected to evaluate the stabilizing ability of NADES for phenolic compounds in safflower. Three representative peaks included: hydroxysafflor yellow A (HSYA) (Tr=21.9 min), cartormin (Tr=40.0 min) and carthamin (Tr=72.9 min). The chosen compounds represent a whole range of polarity and include major and important active metabolites in the safflower extract, which should reflect the stabilization ability of NADES for phenolic compounds in terms of polarity. The stability of the safflower extract and carthamin standard in NADES were investigated at ambient conditions in daylight over a 15-day period.

The results show that the degradation of the carthamin standard was very different in each solvent (Fig. 5). The stability of carthamin decreased in the following sequence, 90% GCH>75% SuCH=LGH>40% EtOH>PMH=water (Fig. 5a). The carthamin in the safflower extract solution showed a similar stability profile to the carthamin standard after 15 days (Fig. 5b). The

degradation of carthamin was found to be 25% in 90% GCH three days after treatment, and 60% in water and 40% ethanol. Thus, the stability of carthamin is markedly improved in 90% GCH ascompared with that in water and EtOH (40%).

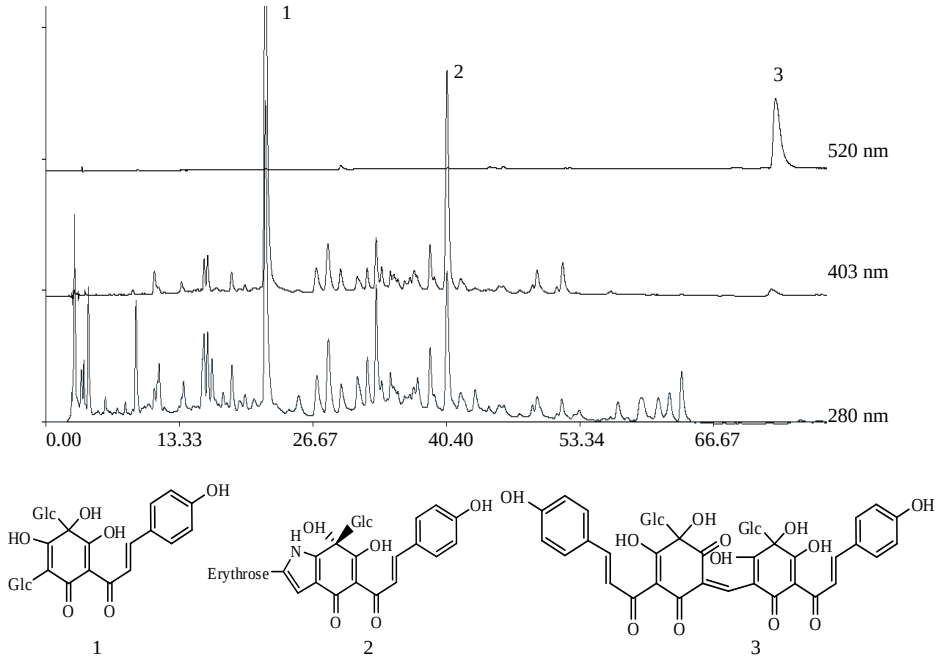

Fig. 4. HPLC chromatograms of safflower extract at 280, 403, and 520 nm (including **1**, hydroxysafflor yellow A; **2**, cartormin and **3**, carthamin).

The behaviour of HSYA and cartormin is very different to carthamin. HSYA was stable in 90% GCH and 75% SuCH, and showed a 5% degradation in water and 40% ethanol in 15 days. However, it degraded rapidly in LGH and 75% PMH (Fig. 5c). Cartormin was also stable in 90% GCH and 75% SuCH, exhibited around 10% in water and 40% ethanol in 15 days, and also degraded dramatically in LGH and 75% PMH (Fig. 5d). Light has been reported to affect the stability of a safflor yellow extract (main components including HSYA and cartormin) in buffer solution (Fatahi *et al*., 2009); the main components of safflor yellow, HSYA and cartormin, however, are more stable in 90% GCH and 75% SuCH than in water and 40% ethanol in our studies. This indicates that 90% GCH and 75% SuCH are far better solvents for storage of phenolic compounds in safflor yellow at ambient than water and 40% ethanol. Ultimately, SuCH and GCH are promising as solvents and color protectors for safflower yellow extracts when applied in the food or pharmaceutical industry.

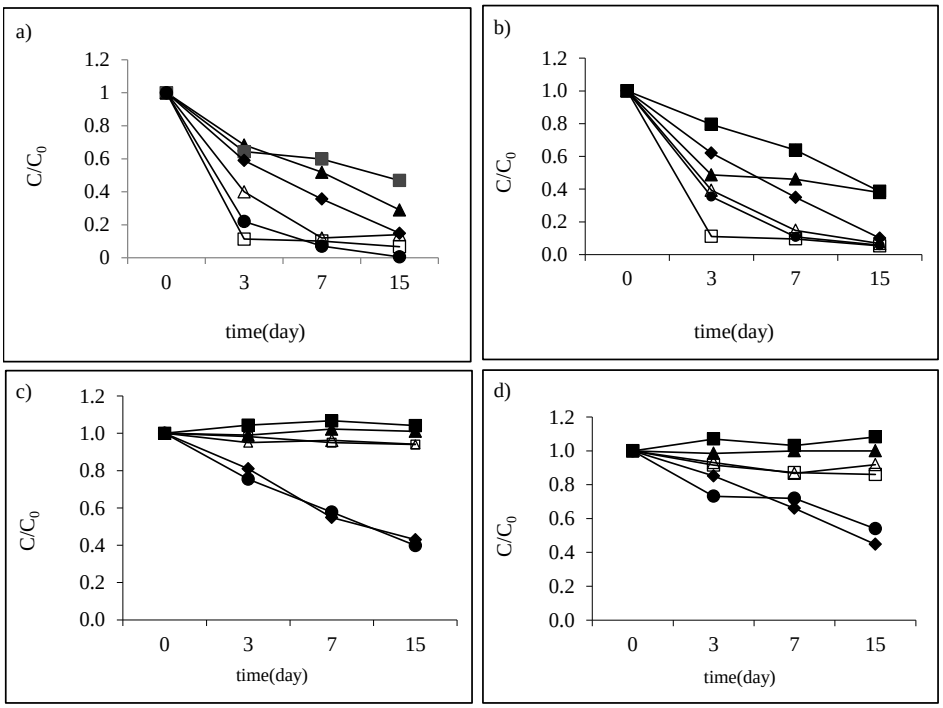

Fig. 5. Stability of three typical phenolic compounds from the safflower extract in different natural deep eutectic solvents at ambient conditions with sunlight in 15 days, compared with in water and 40% ethanol (**a**) carthamin standard, **b**) carthamin in extract, **c**) hydroxysafflor yellow A in extract, and **d**) cartormin in extract). (■: 90% GCH; ▲: 75% SuCH; ♦: LGH; ●:75% PMH; Δ: 40% ethanol; □: water) (*n*=3).

Our results revealed that carthamin (red pigment), HSYA and cartormin (major components of safflor yellow) are more stable in GCH and SuCH than in PMH, LGH, water or 40% ethanol. Sugars (xylose, glucose, sucrose, fructose, lactose) were reported to protect the color of safflower yellow B (a major component of safflor yellow) at high temperature (Saito and Murata, 1994). Therefore, in the first place, it could be possible that the sugar component in NADES may play an important role in stabilizing the safflower extract in ambient conditions, probably due to hydrogen bonding with solutes. Secondly, pH is reportedly an important factor for the degradation of safflower extracts (Saito and Mori, 1994). Safflor yellow is more stable in acidic (pH 2-6) than basic solutions (Yoon *et al*., 2003; Zhu and Pan, 2007; Fatahi *et al*., 2009). LGH and PMH contain acidic ingredients and they are thus more acidic than SuCH and GCH when diluted with 90% (v/v) water. However, HSYA and cartormin are much more stable in GCH and SuCH than in LGH and PMH. Thus, there is no relationship between the stability of HSYA and cartormin and the presence of acids in NADES. Carthamin, on the other hand, is more stable in GCH and SuCH than in PMH and LGH, which is in agreement with the report that carthamin is more stable in basic than acidic aqueous solution (Kim

and Paik, 1997; Fatahi *et al*., 2009). Lastly, the direct relationship between the stabilizing ability of NADES and their high viscosity is confirmed. The viscosity of NADES is greatly affected by the water content (Dai *et al*., 2013) so that the water content may affect the stabilizing ability of NADES, as demonstrated below (section 3.5). All things considered, safflower extracts are more stable in high viscous non-acid NADES.

3.5 Stability of carthamin in NADES with different water contents

The effect of the water content (in the form of water percentage) on the stability of carthamin was investigated in two NADES (SuCH and PMH) at 4 ^{o}C and -20 ^{o}C. Both are highly viscous (Dai *et al*., 2013), but have a different compositionand acidic properties after dilution. Their viscosity is affected by the water percentage and temperature (Dai *et al*., 2013). As discussed previously the viscosity of NADES affects their solubilization ability according to their dilution with water (Dai *et al*., 2013) and may also affect their stabilization ability. Therefore, it is necessary to evaluate the effect of the water content on the stability of compounds in NADES for their application in dissolving and storage of compounds.

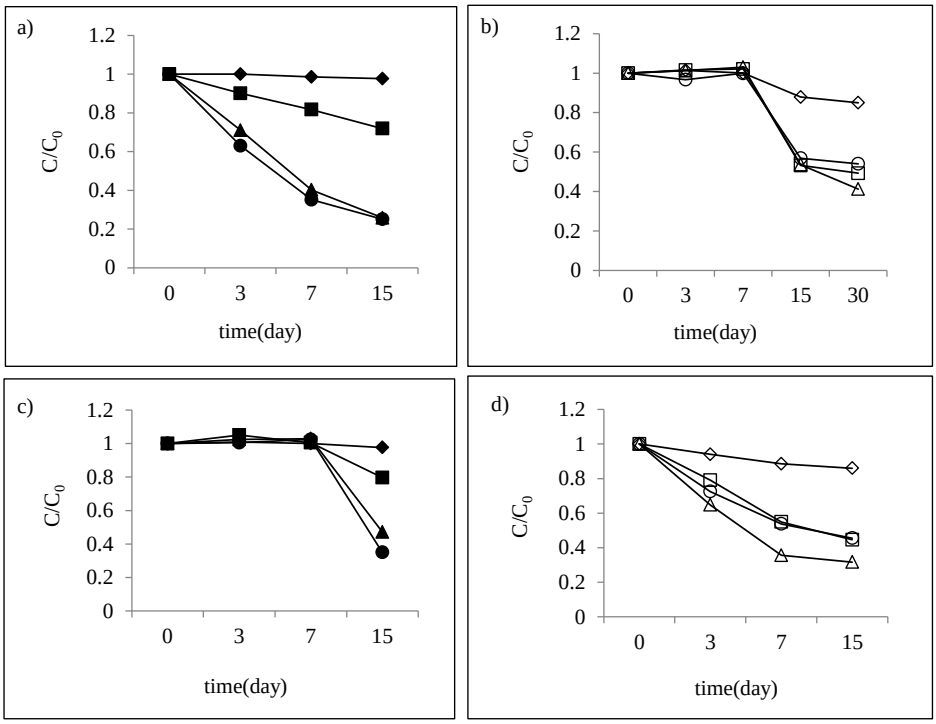

Fig. 6. Stability of carthamin standard in sucrose- choline chloride (SuCH) and proline- malic acid (PMH) with different percentage of water in the dark at at 4 ^{o}C (**a**, **b**) and a-20 ℃ **(c**, **d**). (♦: 100% SuCH; ■: 75% SuCH; ●: 50% SuCH; ▲: 25% SuCH; ◊: 100% PMH; □: 75% PMH; ○:50% PMH; Δ: 25% PMH) (*n*=3).

The results showed that the water content of NADES plays an important role on the stability of carthamin. At 4 ^{o}C, carthamin was stable in SuCH, but less stable in water-diluted SuCH, with a decreasing stability from SuCH: H_2O (3:1), to SuCH: H_2O (1:1), and SuCH: H_2O (1:3) (Fig. 6a). In PMH, the stability of carthamin decreased in the following sequence, PMH > PMH: H_2O (3:1) = PMH:H_2O (1:1) > PMH:H_2O (1:3) (Fig. 6b). After 15 days, the level of carthamin in pure SuCH was still the same, but decreased a 25% in SuCH:H_2O (3:1), and a 75% in both SuCH:H_2O (1:1) and SuCH:H_2O (1:3). Therefore, the stabilization ability of NADES increases with increasing viscosity (low water content). At -20 ^{o}C, the effect of the water content in NADES on the stability of carthamin was lower and became visible after 7 days storage (Fig. 6c, d). Thus, lower water content in highly viscous NADES at low temperature is good for the stability of carthamin.

3.6 Mechanism of the stabilizing ability of NADES for phenolic compounds

FT-IR was recorded for a typical phenolic compound, quercetin, dissolved in GCH (Fig. 7). The spectra show different absorption bands of the C-OH and aromatic ring as well as the carbonyl group from quercetin in solid state and dissolved in GCH. The stretching vibration absorption band of the C-OH of quercetin shifted from 1168 cm^{-1} to 1164 cm^{-1}, indicating that the hydroxyl groups in quercetin donate protons to form hydrogen bonds with solvent molecules. The deformation vibration absorption bands of the C-OH in quercetin shifted from 1355 cm^{-1} to 1369 cm^{-1} and from 1210 cm^{-1} to 1200 cm^{-1}, which confirms the formation of new hydrogen bonds between quercetin and GCH and also implies that quercetin has a different conformation in GCH than in solid state. The shift of the characteristic band of the aromatic ring of quercetin from 1615 and 1600 confirmed the structure deformation of quercetin in GCH (Heneczkowski *et al.*, 2001). In addition, the downward shift of C=O from 1669 cm^{-1} to 1654 cm^{-1} indicates H-bonding (C=O---HO) between the carbonyl group of quercetin and solvent hydroxyl groups (glucose and choline). All the above spectral characters of quercetin reveal the existence of multi H-bond interactions between quercetin and NADES, which is in agreement with the interaction signals in the NOESY spectrum of quercetin in XoCH (Dai *et al*., 2013).

The H-bond interactions between solute and molecules of NADES provide an explanation for the high stabilizing ability of the sugar-based NADES such as SuCH. It was reported that the water extract of safflower is stable in acid conditions (pH 2-6), in which most phenolic compounds are in the neutral form. Further studies showed that sucrose, glucose, starch and the common ions Ca^{2+}, Mg^{2+} and Zn^{2+},can improve the stability of the extract (Zhu and Pan, 2007). All these examples confirm the conclusion that the formation of hydrogen bonds or chelates can stabilize the structures of the phenolic compounds.

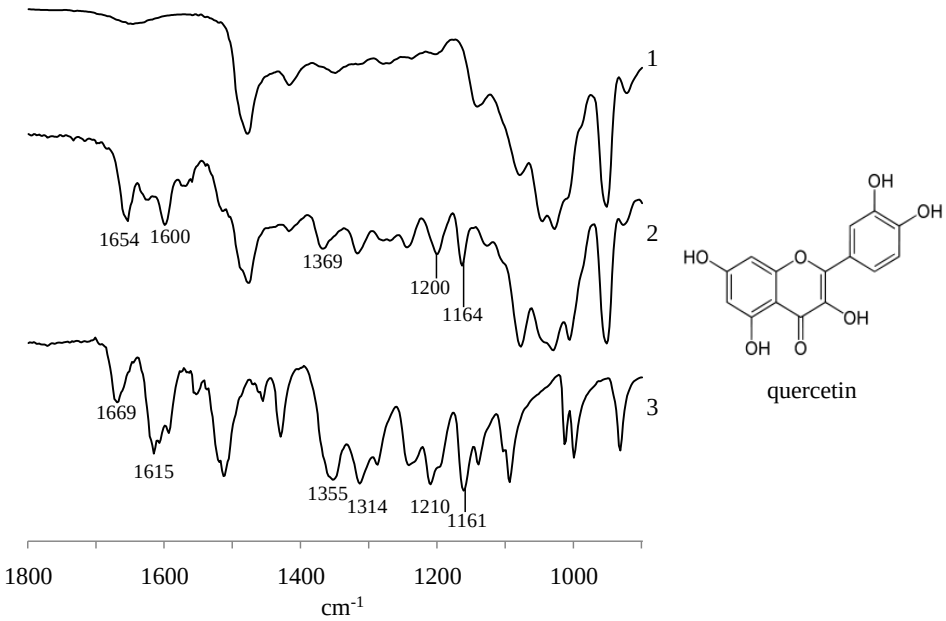

Fig. 7. FT-IR spectra of **1**) glucose-choline chloride **2**) quercetin in glucose-choline chloride; and **3**) quercetin.

All things considered, we hypothesize that the NADES solution of the safflower extract are, in fact, liquid crystals in which the dye molecules are fixed in the crystals. Degradation by oxidation only thus occurs on the surface of the liquid crystals. Melting the crystals by increasing the temperature or adding water allows the phenolics to diffuse freely through the system and thus increased oxidation will occur.

The water extract of safflower is characterized by a high amount of glucose, fructose and choline, as shown in the ^{1}H NMR spectrum of the water extract of safflower. The red and yellow pigments in safflower are accumulated in flowers exposed to the sunlight. However, pure carthamin is unstable in water solution. These findings provide indirect evidence, therefore, to support our hypothesis that NADES in plants are involved in the storage of various non-water soluble metabolites or unstable compounds in cells.

4. Conclusions

In this study, some typical NADES were demonstrated to be better solvents for the stabilization of phenolic compounds than general solvents. The NADES improve the stability of carthamin under various conditions such as high temperature, light and storage time, if compared to water and 40% ethanol. Furthermore, the higher color stability of carthamin as well as of the other two

phenolic compounds in the safflower extract was also observed in NADES when exposed to sunlight at ambient conditions.

The high stabilizing ability of NADES seems to be correlated with the strong hydrogen bonding interactions between solutes and solvent molecules. High viscosity of sugar-based NADES with low water contents allows stable molecular interactions and plays an important role in the stabilizing effect of NADES for phenolic compounds. NADES are like liquid crystals in which the dye molecules are fixed in the crystals.

The high stability of the three typical phenolic compounds from safflower in sugar-based NADES throws new light on the stabilizing ability of NADES for phenolic compounds. Further studies on the stabilization effect in other sugar-based NADES and NADES made of organic acids may not only provide further understanding of the principle of the stabilizing ability of NADES, but also lead to novel applications of NADES in the food and pharmaceuticals industry.

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