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Chapter 7

Natural Deep Eutectic Solvents as new extraction media for phenolic metabolites in *Carthamus tinctorius* L.

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Abstract

Developing green solvents with low toxicity and low cost is an important issue for chemical industry, particularly for pharmaceuticals, food and cosmetics. Synthetic ionic liquids and deep eutectic solvents have received considerable attention due to their negligible volatility at room temperature, high solubilization ability and selectivity. However, the potential toxicity of the synthetic ionic liquids and solid state at room temperature of most deep eutectic solvents hamper their applications as extraction solvents. In this study a wide range of recently discovered natural ionic liquids and deep eutectic solvents, composed of primary metabolites of living organisms, called Natural Deep Eutectic Solvents (NADES), were investigated for the extraction of phenolic compounds of diverse polarity. NADES present many advantages including low cost, simple preparation, low or negligible toxicity profile and sustainability in view of environment and economics benefits. They are stable liquids even below zero ^{o}C, and show high solubilization strength for a wide range of compounds, especially for poorly water-soluble compounds due to their unique physicochemical properties. All those features suggest their potential as green solvents for extraction. Safflower (*Carthamus tinctorius* L.) was selected as a case study because its aromatic pigments covers a wide range of polarities. Experiment with different NADES and multivariable data analysis demonstrated that the extraction ability for both polar (such as hydroxysafflor yellow A, cartomin) and less polar compounds (carthamin and five stereoisomers of tri-*p*-coumaroylspermidine) was greater with NADES than conventional solvent. Water shows high ability for extraction of polar compounds and ethanol for less polar ones. A parameters optimization study reveals that the water content in NADES has the greatest effect on the yield of phenolic compounds. A high concentration of NADES is suitable for less polar

compounds but for more polar compounds low concentration is preferable. Most major phenolic compounds were recovered from NADES with a ratio around 75%-97%, with similar chemical profiles of phenolic compounds as the 40% ethanol extract. Compared with conventional organic solvents, this study provides a greener approach for the extraction of phenolic compounds, implying that NADES have the potential to replace conventional organic solvents in health related areas such as food, pharmaceuticals, and cosmetics.

Key words: *Carthamus tinctorius* L., Asteraceae, natural ionic liquids and deep eutectic solvents (NADES), extraction ability, recovery, phenolic compounds

1. Introduction

Conventional organic solvents are widely used in preparation bioactive components from natural products resources in pharmaceutical, food, and cosmetic industry. The wide range of polarities and physical properties of cellular metabolites make it impossible to extract all metabolites from biomass in a one-step process with one single solvent (Verpoorte et al., 2006). Thus, a wide range of solvents of different polarities is required for the extraction, separation, purification, and administration of various chemicals. So far, alcohols, chloroform, and ethyl acetate are generally applied to this purpose. However, the use of large amounts of organic solvents may cause severe pollution of the environment, and result in organic impurities in extracts, requiring special assays in quality control of extracts (Puranik *et al*., 2009).

With the aim of developing environmental friendly solvents, ionic liquids (ILs) have received increasing attention because they have negligible vapor pressure at room temperature (Welton 1999; Visser *et al*., 2000). Compared with molecular liquids, ILs are a class of organic salts with a low melting point (<100 ^{o}C). Synthetic ILs possess attractive properties such as negligible volatility, tunable physicochemical properties (Huddleston *et al*., 2001), ability to dissolve a wide spectrum of solutes, and special tailor-made selectivity in extraction and separation (Yao *et al*., 2009; Ragonese *et al*., 2011). They have mainly been used in organic chemistry (Welton, 1999) and electrochemistry (Macfarlane *et al*., 2007). However, the application of synthetic ILs as solvents for extraction in pharmaceutical industry is limited because of high toxicity of some ingredients (Docherty and Kulpa, 2005; Quijano *et al*., 2011), their irritation properties, high costs of synthesis of the components, and tedious preparation procedures. Ionic liquids have been used for extracting some active compounds from plant materials including alkaloid (Ma *et al.*, 2010), phenolic compounds (Du *et al.*, 2007), essential oils (Bica *et al.*, 2011) and shikimic acid (Usuki *et al*., 2011).

Deep eutectic solvents (DES) are another type of solvents with similar physical properties (Abbott *et al*., 2007). The solvents are composed of a

mixture of organic compounds and have a melting point much lower than either of the individual component. A series of DES are reported with different components, such as choline, urea (Abbot *et al*., 2003), organic acids (Abbot *et al*., 2004), sugars (Imperato *et al*., 2005). Compared with ILs, DES show some advantages, especially considering their lower environmental and economic impact, e.g. biodegradablity, pharmaceutical accepted toxicity, low cost, and simple preparation methods. They have already been used as solvents to extract DNA and for enzymatic reactions (Gorke *et al*., 2008; Zhao *et al*., 2011). However, the high viscosity and solid state of most DES at room temperature hamper their applications for extraction (Abbott *et al*., 2003, 2004; Imperato *et al*., 2005) and there is still no report on their use for extracting active compounds from plant materials.

In previous studies, we have found a series of natural ionic liquids and deep eutectic solvents, composed of primary metabolites common in living cells, called natural deep eutectic solvents (NADES). In certain Molar ratios, these NADES (e.g. equimolar) show strong intermolecular interactions and may sometimes include water as its ingredients (Choi *et al*., 2011). Apart from sharing all the advantages of reported ILs and DES, NADES possess better properties for extraction, e.g., liquid state even below 0 ^{o}C, adjustable viscosity, and last but not least, sustainability (Dai *et al*., 2013). NADES contain components abundant in our daily foods and thus they are cheap, sustainable, and safe. Interestingly, some NADES show very high solubilization ability for both non-polar and polar compounds, and some metabolites show significantly higher solubility in NADES than in water (Choi *et al*., 2011; Dai *et al*., 2013). This indicates the great potential for NADES as solvents in the extraction of valuable secondary metabolites for their application in the food or pharmaceutical industry

Despite the extensive research on NADES, there is still a lack of information on practical issues related to their application as extraction solvent, such as their effiency, the optimal concentration of NADES, the recovery of compounds from NADES extracts. The latter is particularly challenging considering the inherent low vapor pressure of NADES which makes it difficult to recover solutes from the NADES solution. In this paper, we deal with three aspects of NADES as an extraction solvents: i) the extractability of NADES for natural dyes (phenolic compounds) of diverse polarities, ii) the optimization of the extraction parameters for these phenolic compounds with three typical NADES, and iii) the recovery of the phenolics from the NADES extract. As an example, safflower (Flos carthami), the corolla from *Carthamus tinctorius* L. (Asteraceae), was selected because of its high amount of phenolic compounds of a broad range of polarities and also its application as medicine for promoting blood circulation (Kazuma *et al*., 2000; Zhou *et al*., 2008). It contains yellow (hydroxysafflor yellow) and red pigments. Hydroxysafflor yellow A (HSYA), cartormin and carthamin are the main pigments in safflower used as dye for food and cosmetics. HSYA is also the major active component of safflower

(Watanabe *et al*., 1997; Jin *et al*., 2008). Another group of compounds present in safflower with anti-human immunodeficiency virus infection and anti-depressive activities are tri-p-coumaroylspermidines (Ma *et al*., 2001; Jiang *et al*., 2008; Zhao *et al*., 2009). In addition, different flavonoids, including rutin, quercetin, and their glucosides are reported (Wang *et al*., 2005; Kazuma *et al*., 2000). To investigate the extractability of NADES for those diverse components, HPLC fingerprint and multivariate data analysis, particularly principal components analysis (PCA) were employed to obtain an overview of extract profiles.

2. Material and methods

2.1 Plant material

Safflower was bought from Xinjiang province in China. The plant material was identified by one of the authors, Dr. Young Hae Choi, and a voucher specimen (NPL-carthamus-0913) was deposited in the Natural Products Laboratory, Institute of Biology, Leiden University. The dry plant material was ground into powder in a blender with liquid nitrogen.

2.2 Chemicals and reagents

Ethanol of analytical grade and acetonitrile of HPLC grade were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was deionized water. Malic acid, lactic acid, proline, sucrose, glucose, fructose, 1,2-propanediol, sorbitol, and choline chloride were purchased from Sigma (St. Louis, MO, USA). Macroporous resin Diaion® HP-20 from Supelco (Bellefonte, PA, USA) was used in this study. Silica gel from Sigma-Aldrich (St. Louis, MO, USA) and Sephadex^{TM} LH-20 from GE Healthcare Bio-Sciences (AB, Uppsala, Sweden) were used.

2.3 Natural deep eutectic solvents preparation

All NADES including lactic acid-glucose (LGH); proline-malic acid (PMH); sucrose-choline chloride (SuCH); glucose-choline chloride (GCH); sorbitol-choline chloride (SoCH); 1,2-propanediol-choline chloride (PCH); and fructose-glucose-sucrose (FGSH) were prepared with our reported method in chapter 2.

2.4 Extraction with different solvents

Extraction was performed in a sealed bottle with 100 mg plant material and 1.5 mL solvent, heating and stirring at 40 ^{o}C for 1 h. The sample was transferred into an eppendorf tube and centrifuged at 1300 rpm for 20 min. Then the suspension solution was filtered through a 0.45 μm cellulose acetate filter and diluted with same volume of water. Each extraction was in triplicate.

2.5 Extraction parameter optimization

The extraction parameters of the three NADES with high extractability, SUCH, LGH, and PMH, were optimized. These includes ratio of material weight to NADES volume (mg/mL) (40:1, 30:1, 20:1, and 10:1), the water content in NADES (0%, 10%, 25%, 50%, and 75%), and extraction time (30, 60, 90, 120, and 180 min).

2.6 Recovery of compounds from NADES extracts

Samples of 400 mg of powdered plant material was extracted by 6 mL SuCH, PMH and 40% ethanol with the above mentioned extraction method. The extraction solution was divided into two parts, one for HPLC-DADanalysis as the reference, and one for recovery tests of phenolic compounds from the NADES extracts. For the recovery tests, the SuCH and PMH extracts were submitted to the following procedures: 1 mL of the extracts was diluted with 10 mL of deionized water (for SUCH extract, 1% formic acid was added), loaded on an HP-20 column 60 g (height 50 cm), and eluted with enough deionized water (for SUCH extract, 1% formic acid was added) till all the NADES were washed away. The sample was then eluted with 130 mL of 50% ethanol and 260 mL of ethanol. The two ethanolic fractions were combined, dried with a vacuum evaporator, and dissolved with 3 mL of 50% methanol. An aliquot of 1 mL of the diluted solution was analyzed with HPLC-DADand compared with the equally diluted initial extract. Another 2 mL of the extract were dried, dissolved in 0.4 mL methanol-d_4 (99.80% from Cambridge Isotope Laboratories, Andover, MA) and 0.4 mL phosphate buffer (KH_2PO_4, pH 6.0) in deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France), and analyzed by ^{1}H NMR together with 40% ethanol extract with the same dilution. The above experiments were performed by triplicate.

2.7 Isolation of pure compounds

A sample of 100 g of dry ground material was sonicated with two 800 mL portions of methanol for 1 h, filtered and dried with a rotary evaporator. The residue (20.7 g) was partitioned with 90% methanol and n-hexane twice, and the aqueous methanolic fraction was evaporated under vacuum, obtaining a residue of 18 g. This was fractionated on a middle pressure column with 180 g of silica gel (pore size 60 Å, 230-400 mesh) and eluted with 500 mL of *n*-hexane: chloroform (1:1), 1000 mL of chloroform, 500 mL of chloroform: methanol (10:1) and 2000 mL methanol. Each fraction (100 mL) was pooled based on its thin layer chromatography (TLC) profile [stationary phase: 60F254 plate (Merk, Darmstadt, Germany); mobile phase: chloroform-MeOH (8.5:1.5)]. Three fractions containing phenolic compounds - 26 (40 mg), 29-30 (56 mg), 33-37 (127 mg) - were collected. Each combined fraction was purified on a column with 55 g of Sephadex LH-20, eluted with methanol and further purified with semi-preparative HPLC, using a Phenomenex Luna C18 (250×10 mm, 5 μm, Torrence, CA, USA) column and acetonitrile–water (23:77, 3 mL/min) as a

mobile phase. The isolated compounds were dissolved in 1.0 mL methanol-d4 for measurement for their structural elucidation.

2.8 HPLC, NMR and MS analysis

Quantitative HPLC analysis was performed on an Agilent 1200 chromatographic system with a photodiode array detector (DAD) and separated on a HPLC column, Phenomenex Luna C18 (4.6 μm x 250 mm, 5 μm). The mobile phase consisted of water with 0.5% H3PO4 (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 a flow rate of 1.0 mL/min (Wang, *et al*., 2008). Chromatograms were recorded at 520 nm, 403 nm, and 280 nm. The injection volume was 10 μL.

^{1}H NMR spectra, correlation spectroscopy (COSY), *J*-resolved spectra, heteronuclear single quantum coherence (HSQC), heteronuclear multi-bond correlation spectroscopy (HMBC), and attached proton test ^{13}C NMR (APT) of four purified compounds were recorded at 25 ^{o}C, on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz (^{1}H) and 150.13 MHz (^{13}C) with MeOH-d_4 as the internal lock. All the parameters were the same as those described in our previous report (Ali *et al*., 2011).

Mass spectra were measured by an ESI/TOF/MS. The operating conditions of the ESI ion source (Jeol, Tokyo, Japan) coupled to a JMS-T100TD (AccuTOF-TLC) in the positive ion modes were a discharge needle voltage of 2000 V, nebulizing nitrogen gas flow at 1 L/min. The first orifice lens was set to 100 V and Ring lens voltage was set to 13 V. The TOFMS was set with a peak voltage of 2500 V, a bias voltage of 29 V, a pusher bias voltage of -0.76 V, and a detector voltage of 2300 V.

2.9 Data analysis

The areas of eight representative peaks in the HPLC-DADchromatograms from triplicates were subjected to PCA with the Pareto scaling method using the SIMCA-P software (verion12.1, Umetrics, Umeå, Sweden). These peaks corresponded to compounds of the whole range of polarities present in the HPLC-DAD chromatograms. Analysis of variance (ANOVA) was performed with SPSS software (version 14.0, Chicago, IL, USA) using the peak area in the HPLC chromatogram and P values ≤ 0.1 were considered as significant for comparison. The extraction yield was calculated on the basis of the peak area of the selected peak in the HPLC chromatograms of initial extracts. The recovery yield (w%) is calculated with the peak area of selected peaks in HPLC chromatograms as follows:

$W\% = (A_{rec}/A) \times 100\%$.

where A_{rec} is the peak area of a compound in the chromatogram of the recovered mixture; A is the peak area of the same compound in the chromatogram of extraction solution.

3. Results and discussion

3.1 Comparison of the extractability of safflower polyphenols with NADES, water and ethanol

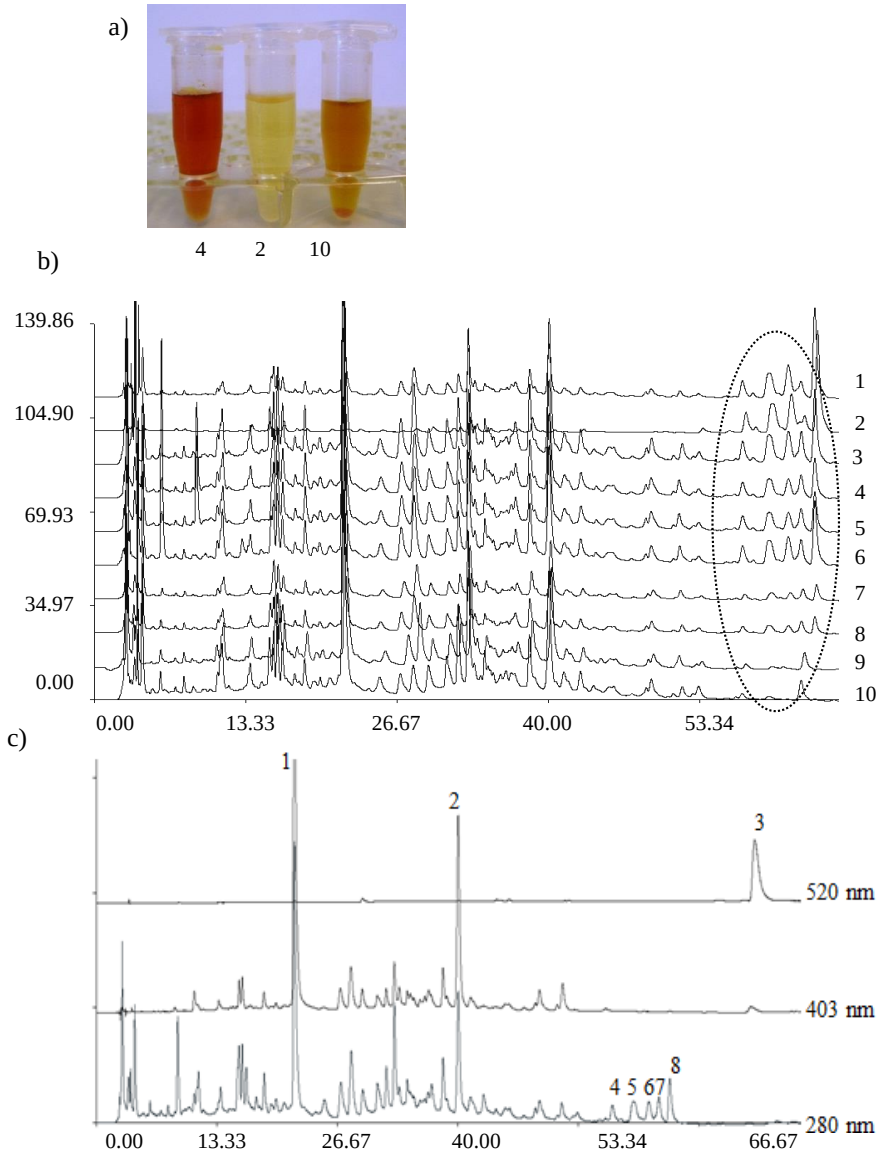

Fig. 1. Safflower extraction solutions **a**) picture of the extraction solutions; **b**) the HPLC-DAD chromatogram profiles of extractions with different natural deep eutectic solvents at 280 nm (1, 1,2-propanediol-choline chloride; 2,

ethanol; 3, 40% ethanol; 4, sucrose-choline chloride; 5, lactic acid-glucose; 6, proline-malic acid; 7, glucose-choline chloride; 8, sorbitol-choline chloride; 9, fructose-glucose-sucrose; 10, water); **c**) HPLC-DAD chromatograms of the extraction in proline-malic acid at three different wavelengths (the labeled compounds are 1, hydroxysafflor yellow A; 2, cartormin; 3, carthamin; 4, N^1, N^{10}, N^5-(*Z*)-tri-*p*-coumaroylspermidine; 5, N^1-(*E*)- N^5N^{10}- (*Z*)-tri-*p*-coumaroyl spermidine; 6, N^1, N^{10}, N^5-(*E*)-tri-*p*-coumaroylspermidine; 7, stereoisomer of tri-*p*-coumaroylspermidine; 8, N^1, N^{10}, N^5-(*E*)-tri-*p*-coumaroylspermidine).

Seven NADES with different polarity, viscosity, composition, and solubilization ability were selected in this study: LGH, PMH, SuCH, GCH, SoCH, PCH and FGSH. NADES with different compositions have different physical properties (Dai *et al*., 2013), and consequently have different solubilizing ability for phenolic compounds.

The major disadvantage of NADES is their high viscosity if compared with conventional solvents. Viscosity is known to restrict the efficiency of NADES as extraction solvents since it results in slow mass transfer. To solve the problem, extraction conditions were adjusted to reduce the viscosity of NADES and thus improve the yield. Different NADES have very different viscosity, but in all cases it can be reduced by the addition of a certain amount of water (Dai *et al*., 2013). Thus, 75% (v/v) SuCH, 75% FGSH, 75% PMH, 90% GCH were used in this study. Another variable known to affect viscosity is temperature, and using 40 ^{o}C as the extraction temperature resulted in increased yields as viscosity decreased. Lastly, in an effort to increase the diffusion rate of the compounds in the liquid with an external force, mechanical agitation was used instead of ultrasound. This resulted in a higher efficiency. Thus, phenolic metabolites were extracted from safflower by agitation of 100 mg of plant material in 1.5 mL solvent at 40 ℃ as described in the experimental section. Given the high viscosity of the resulting extracts samples were centrifuged before filtration.

Clear differences were found in the extractability of compounds from safflower with the tested NADES, water, and ethanol, which were reflected in the color of the obtained extracts and their HPLC profiles (Fig. 1). The NADES extracts exhibited the most intense color (Fig. 1a). The HPLC fingerprints of NADES extracts showed all the peaks observed in water and ethanol extracts (Fig. 1b). Three NADES, LGH, PMH, SuCH extracted polar compounds: HSYA (retention time (Tr) = 21.9 min) (Jin *et al*., 2008; Sun *et al*., 2012) and cartormin (Tr = 40.0 min) (Jin *et al*., 2008; Yin and He, 2000), as well as less polar compounds: carthamin (Tr = 72.9 min, identified with reference) and five stereoisomers of tri-*p*-coumaroylspermidine (Tr = 57.1 - 63.5 min) (Fig. 1c). The identification of each peak was confirmed by comparing NMR and MS data with previous papers and 2D NMR spectra (Jiang *et al*., 2008; Zhao *et al*., 2009). Compounds corresponding to four peaks were isolated and identified as N^1, N^{10}, N^5-(*Z*)-tri-*p*-coumaroylspermidine (peak 4), N^1-(*E*)-N^5N^{10}-(*Z*)-tri-*p*-coumaroyl spermidine (peak 5), N^1, N^{10}-(*E*)-N^5-(*Z*)-tri-*p*-coumaroyl spermidine (peak 6), N^1, N^{10}, N^5-(*E*)-tri-*p*-coumaroyl spermidine (peak 8) (Fig. 2). Peak 7

(a mixture of two compounds) and a small peak between peak 4 and 5, all have the same UV max at around 300 nm and the same molecular weight (583) as the above described tri-*p*-coumaroylspermidines, probably corresponding thus to other stereoisomers of tri-*p*-coumaroylspermidine. The ^{1}H and ^{13}C NMR spectra of coumaroylspermidines showed complex signals because of the restricted rotation around the N-C (sp^2) bond in the coumaroylspermidines (Ma *et al*., 2001). The sequence of elution of these compounds was in accordance with their polarity as can be estimated from their extraction ratios in water and ethanol. Tri-*p*-coumaroylspermidines and carthamin were highly extractable in all three NADES and ethanol, while water did not extract these less polar compounds. Moreover, LGH, PMH, SuCH were more efficient than ethanol even for the less polar compound, like carthamin. The chemical profile of some NADES extracts was qualitatively the same as 40% (v/v) ethanol extract that had been reported as the optimal solvent in extracting safflower yellow (Zhang *et al*., 2009).

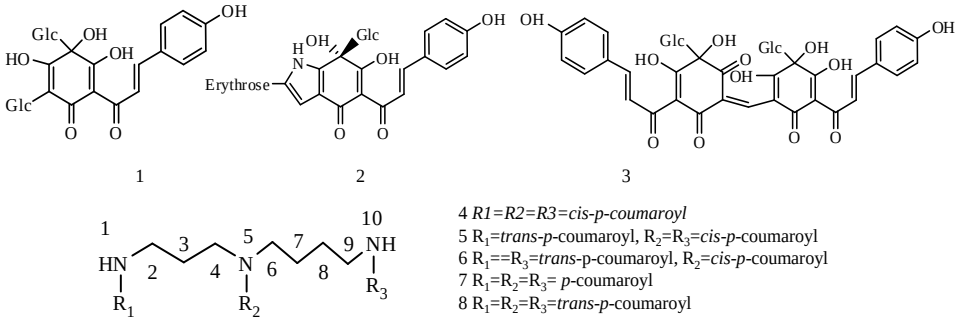

Fig. 2. Chemical structure of target phenolic compounds 1-8 in safflower (the number refers to the same compounds as that in figure 1c).

The score plot of PCA of the first two components (R^2 = 0.94 and Q^2 = 0.89) (Fig. 3a) showed a separation of the extracts into four groups. The PCA confirms the similarity between the extracts with LGH, PMH, SuCH, and the 40% ethanol (Group I), whereas the PCH is similar to the ethanol extract (group II). In the loadings plot of PCA, all metabolites selected from the chromatograms were clustered around group I (Fig. 3b), confirming that solvents in group I had a broad extraction capacity, being efficient for the extraction of both polar compounds (HSYA and cartormin) and less polar ones (carthamin and five stereoisomers of tri-*p*-coumaroylspermidine). In addition, solvents in group II (PCH, EtOH) were efficient in extracting less polar compounds (carthamin and tri-*p*-coumaroylspermidines), while those in group IV (FGSH and water) exhibited a high efficiency for polar ones (HSYA and cartormin), which is in agreement with the reports on the efficiency of water to extract the yellow pigment from safflower (Zhang *et al*., 2007). The extractability of phenolic compounds from safflower is thus higher in SuCH, PMH, and LGH.

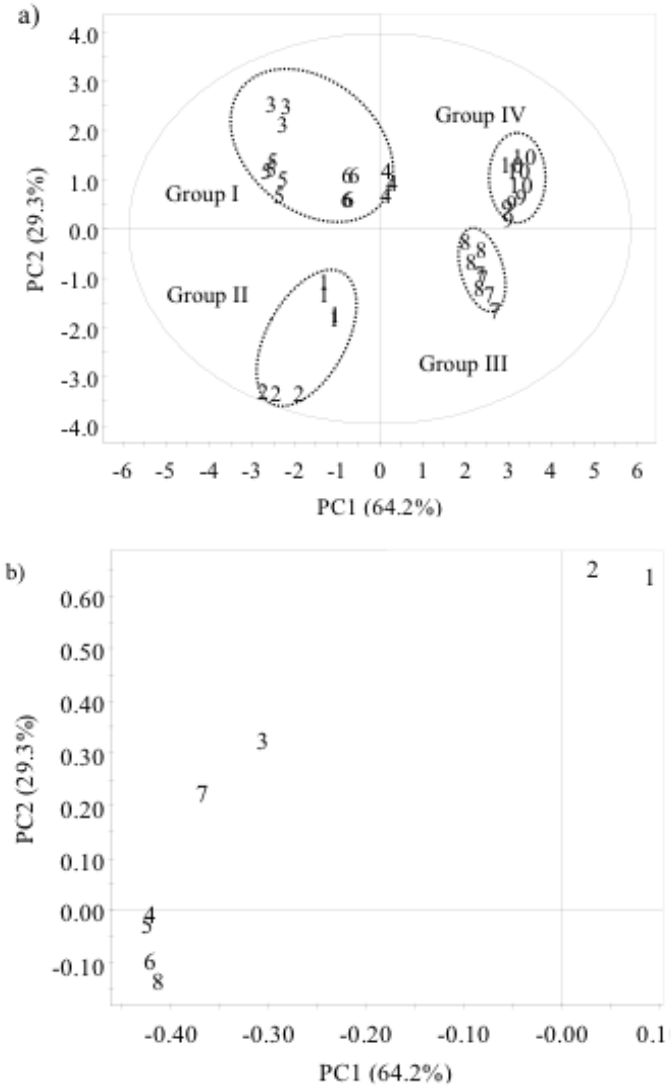

Fig. 3. Score plot **a**) and loadings plot **b**) of principal component analysis of the extracts from safflower with different solvents: (In score plot a, the number refers to the same extract in fig. 1b. In loadings plot b, the number refers to the same peak as in Fig. 1c).

3.2 Optimization of the extraction parameters for NADES with high extraction efficiency

Extraction parameters were optimized using SuCH, PMH, LGH as extraction solvents. The peak areas of HSYA, cartormin and carthamin were used as the criteria to evaluate their extractability. The areas corresponding to peaks of the five tri-*p*-coumaroylspermidines were deleted because they had a retention time close to carthamin and were very small peaks.

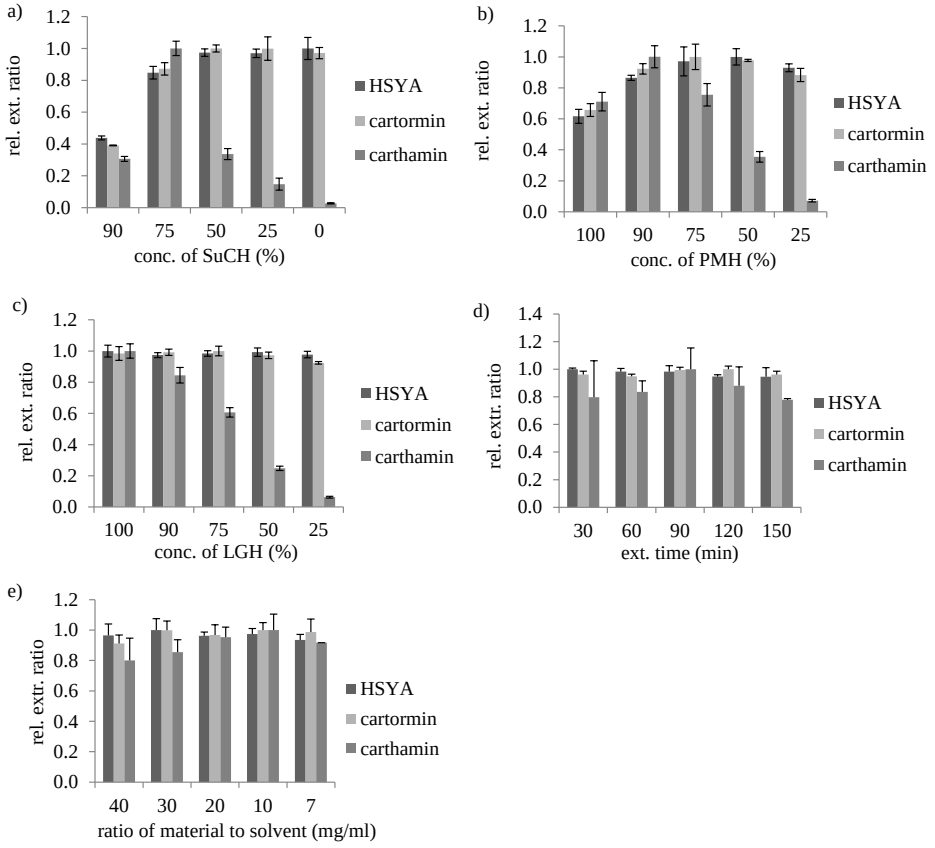

Fig. 4. The effect of water content in NADES on the extraction ability of **a**) sucrose-choline chloride (SuCH), **b**) proline-malic acid (PMH), and **c**) lactic acid-glucose (LGH) and the effect of **d**) extraction time and **e**) ratio of material weight to solvent volume of on the extraction efficiency of three typical phenolic compounds from safflower by proline-malic acid (PMH) for HSYA, cartomin and carthamin. The extraction efficiency is expressed in a relative extraction ratio with the value of a peak area divided by the biggest area of the same peak from different concentrations of the same NADES. The data is expressed in mean ± SD (n=3).

The water content in NADES has a large effect on their extract yield, varying considerably according to the target compounds and the NADES itself (Fig. 4a-c). In the case of HSYA and cartormin, the highest extraction yield was achieved with 50%-100% water in SuCH, with 25%-50% water in PMH and with no addition of water in LGH. For less polar compounds, such as carthamin, the extraction yield was also greatly affected by the water content in NADES. The highest extraction yield of carthamin was reached with 25% (v/v) water in

SuCH, 10% water in PMH, and without water in LGH. The extraction yield of carthamin in SuCH with 25% water was around three times higher than in SuCH with 10% or 50% water. So, in general, NADES with high water content are better for polar compounds and NADES with low water content are suitable for the extraction of less polar compounds. The extraction yield was not significantly affected by any of the other two studied factors: extraction time and ratio of material weight to solvent volume (Fig. 4d-e).

Table 1. The extraction yield of three representative metabolites (hydroxysafflor yellow A (HSYA), cartormin, and carthamin) from safflower with 6 different solvents (The data are the peak areas of each peak in HPLC-DADchromatogram at 403 nm for HSYA and cartormin, and 520 nm for carthamin with 10 μL injection volume, extracted from 90 mg safflower powder with 3 mL solvent and diluted with 2 times water. The data are expressed in mean ± SD (*n*=3).

	extraction yield		
solvents	**HSYA**	**cartormin**	**carthamin**
75%PMH[1]	2813±2	2925±37	134±0
75%SuCH[1]	2680±3	2591±9	152±0
LGH[1]	2244±123	2229±54	235±26
40%EtOH	2611±80	2528±79	182±42
Water	2843±28	2520±19	5±1
ethanol	30±0	13±1	12±0

[1] 75% PMH: 75% (v/v) proline-malic acid in water; 75% (v/v) SuCH: 75% sucrose-choline chloride in water, and LGH: lactic acid-glucose.

Thus, the optimized extraction conditions for these NADES were established as: 1 hour, ratio between material weight and solvent 30 mg/mL). Under these conditions, the relative extract yield of 75% PMH, 75% SuCH, and LGH was compared with that of ethanol, 40% ethanol and water (Table 1). The most efficient extraction solvent proved to be PMH (75%) for HSYA (the same as water and 8% higher than 40% ethanol) and cartormin (14% higher than water and 40% ethanol). In the case of carthamin, LGH showed the highest extraction yield (23% higher than 40% ethanol).

3.3 Recovery test of phenolic compounds from NADES

In order to recover the phenolic compounds from NADES extracts, a chromatographic resin, HP-20, was used. HP-20 (composed of styrene-divinylbenzene) can absorb phenolic compounds while the polar ingredients of NADES can be dissolved and eluted with water. Most phenolic compounds were recovered with ethanol after eluting the polar compounds with water. However, the components of NADES, highly concentrated aqueous solution of the ingredients, as the case with SuCH, can affect the separation process. Using

water, phenolic compounds were eluted together with sucrose from SuCH. However, the addition of 0.1% formic acid to the water increased the retention of phenolic compounds on the column so that they were separated from the components of the SuCH aqueous solution. This approach did not work so well with LGH, since the separation of phenolic compounds was hindered because lactic acid was also attached to the column.

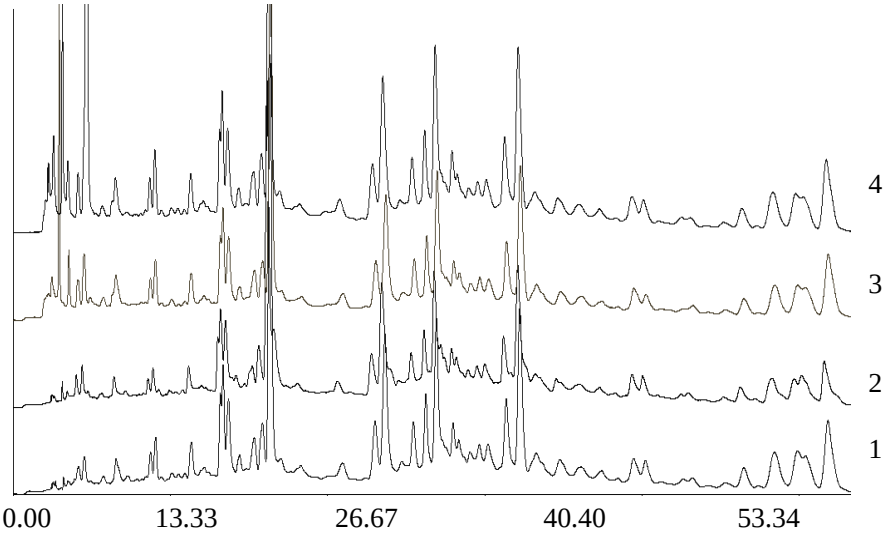

Fig. 5. HPLC chromatogram profiles at 280 nm of safflower extracts **1**) recovered with HP-20 from sucrose-choline chloride (SuCH), **2**) recovered with HP-20 from proline-malic acid (PMH), **3**) 40% ethanol extract, and **4**) PMH extract.

Table 2. The recovery yield of three representative metabolites (hydroxysafflor yellow A, cartormin, and carthamin) in safflower from two NADES (The values are mean ± SD, *n*=3).

	relative yield (%)		
	HSYA	**cartormin**	**carthamin**
75%PMH	92%±9	92%±8	84%±8
75%SuCH	71%±4	86%±4	90%±13

The chromatographic profile of the solutions containing the recovered compounds from NADES (e.g. PMH and SuCH) were qualitatively similar to the 40% ethanol extract except for the solvent peaks, as shown in the HPLC-DAD chromatogram at 280 nm (Fig. 5). The ^{1}H NMR spectra of the recovered extracts confirmed that the components from NADES were successfully separated from the targeted compounds. Furthermore, three replicates showed qualitatively the same chemical profile in HPLC-DAD chromatograms and ^{1}H

NMR spectra. Recovery rates of up to 90% were achieved for polar compounds (such as HSYA and cartormin) (Table 2). For less polar compounds such as carthamin, around 84% recovered from SuCH and 75% from PMH was obtained. It is thus evident that phenolic compounds can be recovered from NADES extracts.

3.4 NADES features affecting their extraction efficiency

The high extractability of phenolic compounds with NADES may be attributed to H-bonding interactions between molecules of NADES and phenolic compounds. In general, the functional groups involved in H-bonding are hydroxyl groups, carboxylic groups and amine groups, all of which are abundant in NADES while hydroxyl groups are obvious available in phenolic compounds. Our former studies showed that obvious interactions exist between quercetin and NADES (Dai *et al*., 2013). The phenolic compounds in safflower are *C*-glucosyl quinochalcone (Jin *et al*., 2008) and flavonoid glycosides (Kazuma *et al*., 2000). Therefore, H-bonding interactions between molecules of NADES and phenolic compounds lead to a high solubility.

The extraction capacity of NADES is also correlated with their physical properties, including polarity and viscosity. PCH has the lowest polarity among all the tested NADES and showed the lowest efficiency for polar compounds, such as HSYA and cartormin, and high extractability for non-polar compounds. Thus, the polarity of NADES has to be considered as an important property affecting their efficiency. Compared with conventional solvents, the high viscosity of NADES is an important feature but it can be decreased by water dilution (Dai *et al*., 2013). SuCH has the highest viscosity and the viscosity is so high that it is difficult to extract compounds from biomass with SuCH. However its dilution with water increased its efficiency, performing much better with a 25% water content than with 10% water content, most likely due to an increased mass transfer rate by the decreasing the viscosity of SuCH.

4. Conclusion

A mixture of natural solid compounds, NADES proved to be efficient solvents for the extraction of phenolic compounds of diverse polarities. These compounds were able to be recovered from NADES with a resin column (e.g. HP-20). These simple, low-cost, green and efficient methods can be applied to the extraction and isolation of natural products from biomaterials. This holds promise for further application of NADES in pharmaceutical, cosmetic and food industry for the extraction and isolation of natural products using fully green solvents.

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