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RESEARCH PAPER

Natural deep eutectic solvents as the major mobile phase components in high-performance liquid chromatography—searching for alternatives to organic solvents

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Abstract

Over the past six decades, acetonitrile (ACN) has been the most employed organic modifier in reversed-phase high-performance liquid chromatography (RP-HPLC), followed by methanol (MeOH). However, from the growing environmental awareness that leads to the emergence of "green analytical chemistry," new research has emerged that includes finding replacements to problematic ACN because of its low sustainability. Deep eutectic solvents (DES) can be produced from an almost infinite possible combinations of compounds, while being a "greener" alternative to organic solvents in HPLC, especially those prepared from natural compounds called natural DES (NADES). In this work, the use of three NADES as the main organic component in RP-HPLC, rather than simply an additive, was explored and compared to the common organic solvents ACN and MeOH but additionally to the greener ethanol for separating two different mixtures of compounds, one demonstrating the elution of compounds with increasing hydrophobicity and the other comparing molecules of different functionality and molar mass. To utilize NADES as an organic modifier and overcome their high viscosity monolithic columns, temperatures at 50 °C and 5% ethanol in the mobile phase were used. NADES are shown to give chromatographic performances in between those observed for ACN and MeOH when eluotropic strength, resolution, and peak capacity were taken into consideration, while being less environmentally impactful as shown by the HPLC-Environmental Assessment Tool (HPLC-EAT) metric. With the development of proper technologies, DES could open a new class of mobile phases increasing the possibilities of new separation selectivities while reducing the environmental impact of HPLC analyses.

Keywords Green analytical chemistry · NADES · Low transition temperature mixtures · Green solvents · Natural designer solvents · Green chromatography

Introduction

Organic solvents generate large amounts of chemical waste that is harmful to the environment and human health while additionally being costly to dispose of. Green analytical chemistry aims at reducing the use of organic solvents, particularly in chromatographic separations [1]. Chromatographic separations are used in the research of new chemicals and in the production of compounds for both their analysis and purification. Of these chromatographic techniques, high-performance liquid chromatography (HPLC) is one of the most used in the world and reversed-phase (RP-HPLC) is the most used mode in HPLC [2]. Solvent selection rules for HPLC have developed over the past six decades, and acetonitrile (ACN) has been consistently employed as the most used organic modifier

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in RP-HPLC, followed by methanol (MeOH) [3]. This has led to the technological development of instruments and columns for HPLC that have been strongly directed to be compatible with these two solvents, particularly ACN [3]. Even during the global financial crisis in 2009, when there was a critical shortage of ACN with significant price increases [4, 5], ACN remained the solvent of choice for most analysts. Few alternative solvents were tested in that period; however, acetone and ethanol provided good results [4, 6]. The current comfortable situation has led to some limitations in the possibilities of separations in HPLC. This situation is deepened by the fact that many regulating agencies require the employment of validated methods which include ACN as a mandatory mobile phase component [7]. From the growing environmental awareness that led to the emergence of the so-called "green chemistry," new research has emerged to find replacements to ACN [8], especially because it is an undesirable solvent from a sustainable point of view [9, 10]. It was estimated in 2008 that there were 130,000 HPLC instruments operating around the world [11], but more recently, a large manufacturer of HPLC instruments estimated that there are currently 200,000. Considering an average consumption of 0.5–1 L of mobile phase per day [3, 12–15], 26,000,000–52,000,000 L of chemical waste is produced every year worldwide. Therefore, finding alternatives for organic solvents in HPLC is a highly important issue from an environmental performance perspective, in addition to the separation performance perspective.

Current approaches to find alternatives to commonly used environmentally impactful organic solvents, such as ACN, involve replacing them with solvents such as ethanol (EtOH) [3, 13] or acetone [16]. Other chromatographic techniques which require little or no organic solvent such as supercritical fluid chromatography (SFC) [17, 18] and superheated water chromatography (SWC) [19] are also undergoing rapid new developments. These chromatographic methods require specialized equipment and columns capable of working at these supercritical or high temperature conditions [12]. Furthermore, SWC struggles to analyze non-polar compounds due to their low solubility in water [20] and even when enough eluotropic strength is achieved, selectivity is a major issue. More recently, the separation of carboxylic acid compounds (naproxen, ibuprofen, and ketoprofen) was achieved with an amine functionalized silica particle stationary phase and a CO_2-modified water mobile phase, demonstrating another type of environmentally friendly chemical analysis [1]. Different approaches used to reduce organic solvents have been the introduction of surfactants [21] or additives to the mobile phase [8]. Some of these additives have included ionic liquids (ILs) [22] and deep eutectic solvents (DES), most of which can be environmentally considered as "green" solvents. However, it is still important to note that most separations reported in the literature still use at least 10% organic solvents such as ACN or methanol (MeOH) [8, 23].

DES are formed by mixing hydrogen bond donor and acceptor compounds together with the resulting hydrogen bonding network producing a substance with a lower melting point than its individual components [24]. Many advantageous characteristics have been described for DES, such as their ability to be formed from a wide variety of compounds, water compatibility, low vapor pressure, non-flammability, biocompatibility, biodegradability, and easy preparation [25]. To date, many different DES have been reported in the literature and it is estimated that around 10^6 DES could be possible [25, 26]. A sub-class of DES made from primary metabolites (e.g., carbohydrates, amino acids, organic acids), which are common in living cells, have recently been developed and coined natural deep eutectic solvents (NADES) [27]. NADES have been shown to be less cytotoxic than their equivalent DES [28, 29]. Multiple studies have shown the use of NADES as extraction solvents [30–34], whereas few studies of NADES have demonstrated their use in analytical separation techniques. DES have previously been used as mobile phase additives in RP-HPLC allowing for enhanced peak shapes and resolution [35, 36]. However, the concentrations of DES used in these studies were below 8 wt% which means there is likely a complete loss of their supramolecular structure resulting in the physicochemical properties of the DES being very different to when there is little or no water present in the DES [37].

Herein, an assessment of the performance of NADES as alternatives to organic solvents in HPLC separations using current technology was conducted. NADES were tested as major mobile phase components rather than simply as an additive in RP-HPLC. To meet this aim, (i) the use of organic solvents was kept to a minimum, (ii) three different NADES of relatively low viscosity were employed as organic modifiers, (iii) their performances were compared to the more commonly used HPLC solvents ACN and MeOH in addition to the greener EtOH both in terms of chromatographic performance and "greenness," and (iv) the potential separations of two mixtures of analytes were used: protocatechuic acid derivatives (PADs) as well as a mixture of compounds (MIX) of varying acidity, molar mass, and functional groups allowing for an assessment of their chromatographic performance.

Materials and methods

Materials

D-Glucose (Glu, USA, ≥ 99.5%), L-ascorbic acid (USA, 99%), and *N*-hydroxysuccimide (98%) were purchased from Sigma. Choline chloride (CC, Brazil, 98%), lactic acid aqueous solution (LA, Brazil, 85–90%), and ethylene glycol (EG, Brazil, 99.9%) were purchased from Exodo Cientifica. HPLC grade MeOH, EtOH, and ACN were obtained from JT Baker (Brazil). 2-Nitroaniline, vanillin, oxytetracycline, 4,6-dinitro-

ortho-cresol, potassium phenoxymethylpenicillin, 2,6-di-tert-butylphenol, and butylated hydroxyanisole (see Electronic Supplementary Material (ESM) Table S1) were purchased from Henrifarma (Brazil). The PADs (see ESM Fig. S1) were synthesized and purified according to De Faria et al. (2012) [38].

Preparation of NADES

The NADES were prepared by weighing choline chloride (CC), ethylene glycol (EG), lactic acid (LA), glucose (Glu), and water (H_2O) to the desired molar ratios. The desired mixtures were accommodated into a Schott bottle and heated to 50–60 °C while stirring for 30–60 min to lead CC:EG 1:3, CC:EG 1:2, and LA:Glu:H_2O 5:1:4 (all molar ratios). The resulting transparent liquids were heated to 70 °C and vacuum filtered before use as a mobile phase component.

HPLC experiments

Chromatographic analyses were conducted in an ultra-high-performance liquid chromatography (UHPLC) system Ultimate3000 (Thermo, Sunnyvale, USA), equipped with a ternary pump model DGP-3600RS and a SRD-3600 solvent rack with degasser, a thermostatted column compartment TCC-3000RS, a diode array detector DAD-3000(RS), and an autosampler model WPS-3000RS. The equipment was controlled by the software Chromeleon v. 6.80. The sampling rate was 25.0 Hz and the analyses were monitored from 200 to 400 nm. The chromatograms were plotted in Origin2017. Since lactic acid absorbs at both wavelength, 245 and 260 nm, the chromatograms with LA:Glu:H_2O 5:1:4 as the organic modifier were plotted for an easier visual comparison between mobile phase performances, with the baseline subtracted using Origin2017. The original chromatogram without baseline subtraction is included in the ESM (Fig. S2).

Separations were achieved on Chromolith® RP-18e (Merck) or Onyx® C18 (Phenomenex) monolithic columns (100 × 4.6 mm) that were endcapped. For experiments performed with a pure solvent as the organic modifier, mobile phases were composed of water (A) and ACN or MeOH or EtOH (B) using the following solvent gradient: 5–70% B (0–30 min), 70% B (30–35 min), and 70–5% B (35–40 min). For experiments with NADES, the mobile phases were composed of water (A), EtOH (B), and a NADES (C) using the following solvent gradient: 5% B and 0–65% C (0–30 min), 5% B and 65% C (30–35 min), 5–70% B and 65–0% C (35–43 min), and 70–5% B and 0% C (43–48 min). Separations were conducted at 50 °C with a flow rate of 1.5 mL min^{-1}. Detection was at 245 nm except when LA:Glu:H_2O 5:1:4 was used, it was 260 nm.

PADs (ESM Fig. S1) were dissolved in EtOH:H_2O 50:50 such that each compound was between 0.1 and 1.7 g L^{-1}. A mixture of compounds (MIX, ESM Table S1) was prepared from the following compounds being dissolved in EtOH:H_2O 60:40 together to the final concentrations stated in brackets: L-ascorbic acid (84 mg L^{-1}), 4,6-dinitro-ortho-cresol (12 g L^{-1}), vanillin (0.3 g L^{-1}), potassium phenoxymethylpenicillin (1.0 g L^{-1}), oxytetracycline (0.3 g L^{-1}), 2-nitroanaline (0.1 g L^{-1}), butylated hydroxyanisole (0.5 g L^{-1}), 2,6-di-tert-butylphenol (5.6 g L^{-1}). All samples were filtered through 0.22-μm nylon filters.

The resolution (Rs) for overlapping peaks was estimated according to Snyder [39], whereas for none overlapping peaks, it was calculated according to Eq. 1 as stated in reference [40]:

$$Rs = 1.18\left(\frac{t_m - t_{m+1}}{W_m + W_{m+1}}\right) \quad (1)$$

where t and W are the average retention times and full width at half maximum (FWHM) from replicate experiments with peaks m. The peak capacity (PC) was determined according Eq. 2 as stated in reference [39]:

$$PC = 1 + \left(\frac{t_f - t_i}{W_t}\right) \quad (2)$$

where t_f and t_i are the average retention times from replicate separations of the final and initial peaks in the separation respectively and W_t is the average FWHM of all the peaks in all the separations.

Results and discussion

Finding the experimental conditions

RP-HPLC with a C18 stationary phase is the most commonly used liquid chromatographic mode; therefore, the potential for NADES in RP conditions was explored. Although the UHPLC instrument used could accommodate backpressures up to 1000 bar, it was expected that the major experimental challenge in using NADES as a mobile phase would be the high viscosities so careful selection was made for the chromatographic conditions. A highly porous monolithic column (100 × 4.6 mm) was selected as the stationary phase instead of a packed column, and three NADES with comparatively low viscosities were selected to be tested as the major organic components of the mobile phase. For these NADES, the viscosities were still reported to be between 19 and 37 $mm^2\ s^{-1}$ which is at least one order of magnitude more viscous than traditional organic solvents [24, 41]. In order to reduce the viscosity while not damaging the monolithic column, separations were conducted at 50 °C. Initially, the solvents were preheated to this temperature in a water bath before being pumped into the instrument. As there was no observable

improvement in the chromatographic performance compared to that achieved when only a preheater was installed inside the column oven, this extra pre-heating was not used in the experiments shown. A flow rate of 1.5 mL min^{-1} was selected for all analyses. In early experiments, it was observed that using a 0.13-mm-diameter tubing in the HPLC system before the DAD/UV caused the backpressure to reach 400 bar when the mobile phase composition was greater than 65% of the lowest viscosity NADES used, CC:EG 1:3. There is an exponential increase in the viscosity as the water content of the NADES decreases which is likely due to a stronger H bond network present with a lower water content [37]. Since the backpressure was already greater than the 206 bar recommended by the manufacturer, the narrow bore tubing was replaced with 0.18-mm-diameter tubing. Using wider diameter tubing, the backpressure did not exceed 203 bar when the organic content in the mobile phase was below 70% (65% NADES, 5% EtOH, and 30% water), even for NADES more viscous than CC:EG 1:3. Therefore, with careful selection of tubing, temperature, and mobile phase composition as well as the type of NADES and columns used, it is possible to lower the backpressure such that NADES can be used as an organic modifier.

Although there is no reason not to hypothesize that NADES could completely replace organic solvents in RP-HPLC if a stationary phase was developed for such purpose, it was found that it was necessary to maintain 5% of an organic solvent in the mobile phase to maintain symmetrical peak shapes. We hypothesize that this is because the CC:EG-based NADES used were unable to fully solvate the C18 stationary phases causing the C18 groups to not be completely exposed. By having 5% organic solvent in the mobile phase, it appears that the stationary phase was sufficiently solvated. Therefore, organic solvents could not be completely eliminated from the mobile phase with the NADES tested and 5% EtOH was added as a mobile phase component since it is one of the "greenest" organic solvents [42], thus maintaining a method with a very low environmental impact.

Comparing the performances of NADES and organic solvents

The ability of NADES to elute and to separate compounds of increasing hydrophobicity, such as the PADs, was compared with organic solvents to assess the eluotropic strength and selectivity. When the same conditions were employed, the eluotropic strength order observed was LA:Glu:H_2O 5:1:4 ~ EtOH > ACN > CC:EG 1:2 > CC:EG 1:3 > MeOH (Fig. 1). MeOH was unable to elute all the PADs while all the other organic modifiers including the tested NADES were able to elute them (Fig. 1). The same eluotropic strength order was observed when the MIX was analyzed (Fig. 2). The retention times of the PADs when eluted by LA:Glu:H_2O 5:1:4 was 1–5% different to when ACN was used, thus showing that a

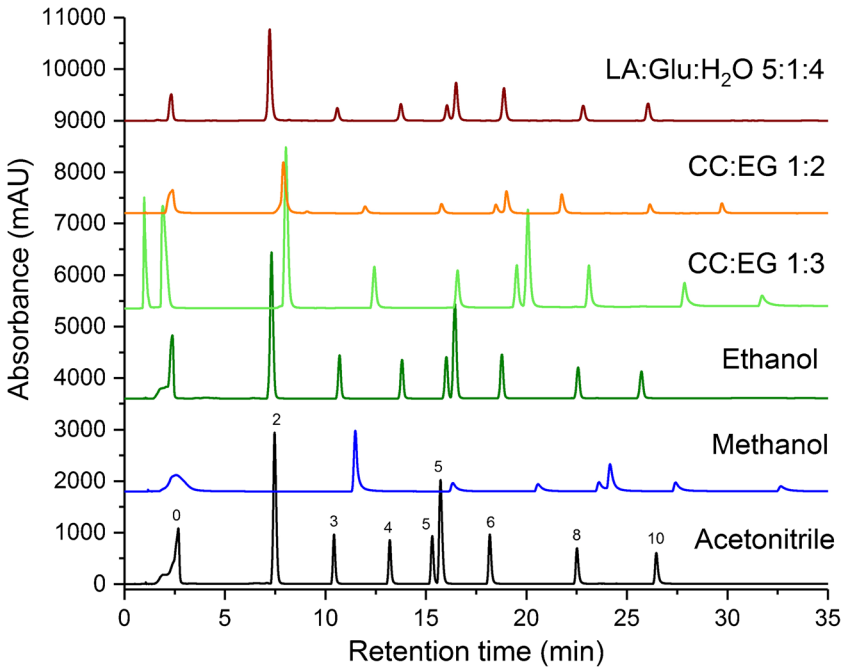

Fig. 1 HPLC-UV chromatograms for a mixture of protocatechuic acid and its eight derivatives (PADs). The numbers correspond to the number of carbons (n) in the derivative chain shown in ESM Fig. S1. Column: C18 RP monolithic column. Mobile phases for experiments performed with an organic solvent: water (A) and ACN or MeOH or EtOH (B) at 5–70% B (0–30 min) and 70% B (30–35 min). Mobile phases for experiments with NADES: water (A), EtOH (B), and NADES (C) at 5% B and 0–65% C (0–30 min), and 5% B and 65% C (30–35 min). Flow rate, 1.5 mL min^{-1}. Injection volume, 10 μL. Detection was at 245 nm except when LA:Glu:H_2O 5:1:4 was used, it was 260 nm and baseline subtracted (see the "HPLC experiments" section)

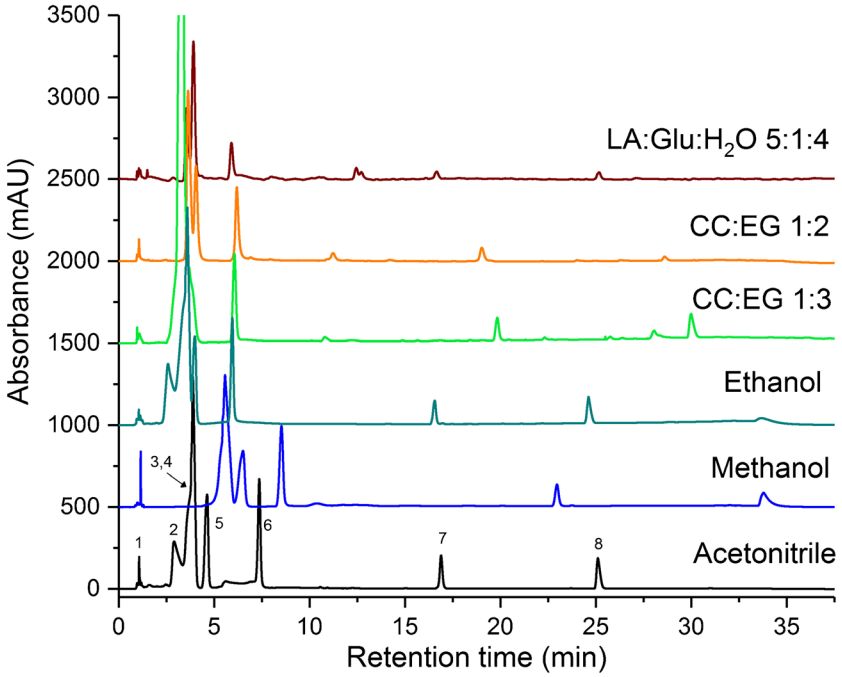

Fig. 2 HPLC-UV chromatograms for a mixture of compounds (MIX): *1* L-ascorbic acid, *2* 4,6-dinitro-ortho-cresol, *3* vanillin, *4* potassium phenoxymethylpenicillin, *5* oxytetracycline, *6* 2-nitroanaline, *7* butylated hydroxyanisole, *8* 2,6-di-tert-butylphenol. Column: C18 RP monolithic column. Mobile phases for experiments performed with an organic solvent: water (A) and ACN or MeOH or EtOH (B) at 5–70% B (0–30 min), 70% B (30–35 min), and 70–5% B (35–40 min). Mobile phases for experiments with NADES: water (A), EtOH (B), and a NADES (C) at 5% B and 0–65% C (0–30 min), 5% B and 65% C (30–35 min), and 5–70% B and 65–0% C (35–43 min). Flow rate, 1.5 mL min^{-1}. Injection volume, 10 μL. Detection was at 245 nm except when LA:Glu:H2O 5:1:4 was used, it was 260 nm and baselined (see the 'HPLC experiments' section)

NADES can have a similar eluting strength to ACN in RP-HPLC. Very similar trends in retention times were observed when NADES or organic solvents were used to separate the MIX (Fig. 2). When the two CC:EG-based NADES are compared, it can be seen that an increase in the CC content (decrease in EG content) increased the eluotropic strength of the mobile phase (Figs. 1 and 2). For the separation of PADs, as shown in Table 1, the median resolutions for all peak pairs were 8.5, 11.2, and 11.4 for MeOH, EtOH, and ACN, respectively, whereas they were 10.1, 10.3, and 11.8 for CC:EG 1:3, LA:Glu:H_2O 5:1:4, and CC:EG 1:2, respectively (calculated using Eq. 1). MeOH led to the lowest median resolution which could be linked to its elution strength since one compound was not eluted with this mobile phase. Only the mobile phase containing CC:EG 1:3 resolved the first two peaks (Fig. 1). These peaks might be the protocatechuic acid and its conjugated base which was not completely resolved when ACN or any other mobile phase components were employed (Fig. 1).

Analyzing the separation of the eight compounds of the MIX (ESM Table S1), the median resolutions for all peak pairs were 2.8, 3.5, and 4.6 for EtOH, ACN, and MeOH, respectively, whereas they were 5.6, 6.7, and 6.7 for CC:EG 1:3, LA:Glu:H_2O 5:1:4, and CC:EG 1:2, respectively (Table 1). However, only separations with mobile phases containing ACN or EtOH led to chromatograms containing eight peaks, but with two overlapping peak pairs (Fig. 2). The minimum resolutions are estimated as 0.5 for both mobile phase components (Table 1). LA:Glu:H_2O 5:1:4, CC:EG 1:3, and MeOH led to seven peaks, with the peak from 4,6-dinitro-ortho-cresol disappearing. CC:EG 1:2 led to six peaks, which evidenced the complete coelution of two pairs of compounds. Overall, the retention behavior and resolutions of all the analytes examined are not significantly different for either NADES or organic solvents when used as the mobile phase.

The FWHM of the peaks of most compounds separated with NADES were smaller than when MeOH was employed as the organic modifier but they were still slightly larger (approximately 5–10%) than when ACN or EtOH were the organic modifier (ESM, Table S2 and Fig. S4). NADES involve more than one molecule, most in a hydrogen bond network and potentially some free solvated molecules when the mobile phase has a high aqueous content. As a NADES is not a single species, the elution mechanism could involve a number of different interactions resulting in the slightly broader peaks. Adsorption of NADES to the stationary phase was observed through peak tailing in chromatograms when the column was subsequently used with organic solvents after having been used with NADES. As expected, the slightly broader peaks observed for NADES compared to ACN in the case of the PAD separation, (ESM Fig. S4) led to slightly lower peak

Table 1 Median peak capacity, resolution, and RSDs of peak retention times, areas, and full widths at half maximum (FWHM) for PADs and MIX when separated with NADES and organic solvent mobile phases with the ranges shown in brackets. Each separation was performed in triplicate

Solvent	Analytes	Median RSD of retention times (%)	Median RSD of peak areas (%)	Median RSD of peak FWHM (%)	Median resolution	Peak capacity
LA:Glu:H_2O 5:1:4	PADs	0.1 (0.1–0.3)	3.5 (0.4–7.6)	1.5 (0.3–2.1)	10.3 (0.0–16.6)	135.7 (135.5–135.9)
	MIX	0.3 (0.1–1.0)	31.8 (15.5–42.2)	9.3 (3.6–13.7)	6.7 (0.0–31.5)	122.4 (118.6–130.9)
CC:EG 1:2	PADs	0.1 (0.1–0.1)	2.7 (0.8–6.1)	0.9 (0.3–10.7)	11.8 (0.0–14.8)	139.4 (137.0–142.3)
	MIX	0.1 (0.1–0.2)	3.6 (1.9–4.4)	1.9 (0.6–7.3)	6.7 (0.0–37.3)	153.6 (150.0–154.2)
CC:EG 1:3	PADs	1.3 (0.8–1.4)	8.3 (2.1–15.0)	5.5 (2.7–10.3)	10.1 (1.8–15.4)	103.3 (100.2–114.0)
	MIX	0.1 (0.1–3.0)	6.6 (2.3–21.4)	1.8 (0.2–7.9)	5.6 (0.0–41.9)	124.9 (120.7–127.0)
Ethanol	PADs	0.2 (0.1–1.3)	2.8 (1.5–5.6)	2.5 (0.4–8.1)	11.2 (0.8–16.6)	141.6 (140.9–146.7)
	MIX	0.1 (0.1–0.2)	5.3 (0.9–13.7)	1.9 (0.1–8.5)	2.8 (0.5–39.5)	96.5 (92.4–96.6)
Methanol	PADs	0.2 (0.1–9.4)	13.7 (0.7–17.2)	15.5 (1.2–32.4)	8.5 (0.0–13.3)	91.8 (80.4–101.1)
	MIX	0.1 (0.1–0.2)	5.0 (2.9–15.8)	2.8 (2.2–10.2)	4.6 (0.4–42.4)	133.2 (123.7–134.8)
Acetonitrile	PADs	0.1 (0.0–0.3)	1.6 (0.5–27.9)	0.4 (0.4–56.4)	11.4 (0.8–17.9)	156.7 (124.8–158.1)
	MIX	0.1 (0.1–0.3)	3.0 (1.5–6.9)	1.0 (0.1–3.7)	3.5 (0.5–37.2)	110.7 (108.6–112.8)

capacities for NADES (156.7 and 139.4 for ACN and CC:EG 1:2, respectively, Table 1). On the other hand, the peak capacity for CC:EG 1:2 was higher than that observed for MeOH (91.8) and very close to that observed for EtOH (141.6) (see Table 1). All compounds had significant peak tailing with MeOH in the mobile phase and the hydrophobic compounds tested exhibited small amounts of peak tailing when CC:EG 1:3 was used. This tailing contributed to increased peak broadening. The majority of peaks eluted by NADES are symmetrical further showing that NADES are suitable eluents in HPLC. Regarding repeatability, the median of the relative standard deviations (RSDs) for retention times, peak area, and FWHM revealed that there is no significant difference when organic solvent or NADES mobile phases were used (Table 1). The RSDs of the peak area when NADES was used are in the same range as when ILs have been used as additives [43]. The RSDs were generally below 0.5% for the peak retention time with the exception of CC:EG 1:3 and MeOH. The higher RSDs for CC:EG 1:3, CC:EG 1:2, and MeOH are most likely due to some of the peaks tailing. The median RSD of peak areas was higher for the MIX when LA:Glu:H_2O 5:1:4 was used as the mobile phase (Table 1). Since LA absorbs UV, this NADES presented a noisy baseline (ESM Fig. S2) even when a higher detection wavelength was used. This was particularly problematic for the MIX as they absorb more at lower wavelengths than the PADs.

Assessing the "greenness" of the mobile phases

There are a number of different metrics available in the literature for assessing the "greenness" of an analytical method, each with advantages and disadvantages [15, 44, 45]. In the case of HPLC methods, we find the HPLC-Environmental Assessment Tool (HPLC-EAT) to be the most appropriate since it can lead to a fine differentiation between HPLC methods [3, 14]. The HPLC-EAT approach gives a score on the method based on how the solvents used affect the environment but also the risk to the operator in terms of health and safety, where the lower the score, the less impact of the method and thus the more "green" it is. The HPLC-EAT score is calculated according to Eq. S-1, which contains individual scores for Safety, Health, and the Environmental (SHE) impact. The SHE scores are determined from a number of different parameters (e.g., acute and chronic toxicity and persistency in the environment) which were developed in references [46, 47]. The SHE scores for common organic solvents are present in the database of the HPLC-EAT software making the determination of the HPLC-EAT score easy to obtain. However, the NADES components used have not been assessed making the parameters of the SHE scores not available in the software. These parameters were determined with data from the Organization for Economic Co-operation and Development (OECD) (ESM, Tables S3-S5). Selecting the value for the parameters in the SHE scores can be difficult in cases where reliable data about that chemical is not readily available. Since some NADES components are not often the focus of toxicological studies, an accurate toxicity may not be available; thus, the most impactful value was used to determine the SHE scores. Therefore, the SHE scores of the NADES are likely overestimated. Another disadvantage of using these scores is that there is no parameter that describes if it is sourced from a renewable source or not. For example, a method employing petrochemical-based ethanol or bioethanol will get the same HPLC-EAT score. Furthermore, in order to calculate the SHE scores, it is assumed that the NADES would have the same impact as the sum of the individual components. Nevertheless, the estimated scores of the NADES still allow a comparison with organic solvents.

The results of the HPLC-EAT metric for the mobile phases used are shown in Table 2. All the NADES mobile phases have a lower score than the organic solvents, CC:EG 1:2 being the greenest mobile phase employed in this work, followed by CC:EG 1:3 (Table 2) because of the difference in the fraction of CC. Indeed, CC has been stated as a green NADES component [26]. ACN clearly has the highest HPLC-EAT score, followed by MeOH and EtOH. Overall, the *S* scores observed for the NADES when compared with the organic solvents were definitive to rank them as the greener mobile phases (Table 2). The low volatility and flammability of NADES have previously been stated as a clear advantage as to why NADES have less environmental impact than organic solvents [8, 26]. The NADES have similar *H* and *E* scores to the organic solvents (Table 2), likely due to the fact that there is limited reliable toxicological data for the components of the NADES. Overall, the HPLC-EAT scores demonstrate that using NADES as a mobile phase provides a "greener" separation method than using traditional organic solvents.

Current limitations and general notes about NADES as a mobile phase component

NADES is definitely a "greener" solvent type than the traditional ACN or MeOH, and the chromatographic performance in terms of resolution, retention, and peak repeatability is similar to what can be achieved with ACN, MeOH, and EtOH. Therefore, NADES can be used to develop a "green" mobile phase; however, there are still a number of challenges to overcome before routine use could be considered. Firstly, the viscosity does make handling NADES, including filtration, more difficult, laborious, and time consuming than organic solvents. This might be facilitated by the development of HPLC modules especially designed for NADES; for example, a high-pressure automated filtration module in the instrument would speed up this step. It was found that modern HPLC pumps were required to reach the entered flow rate as the volume eluted was lower than programmed in an instrument more than 10 years old. However, since modern HPLC instruments continue to improve their ability to withstand high pressures (≥ 1000 bar) and temperatures, factors such as this are beginning to be considered as less of a hurdle for producing "greener" methods [48]. Furthermore, by using appropriate tubing and high temperatures (≥ 60 °C), the backpressure could be considerably lowered. The difficulty does remain that a number of stationary phases are unable to handle these high temperatures or pressures. Recent developments in the production of highly permeable monolithic columns could solve this issue [49].

Another issue that was observed with NADES was the strong interaction of the NADES with the stationary phase. The adsorption reduced the chromatographic performance of any separation when subsequently using that column with a traditional mobile phase using an organic solvent, even after extensive washing. However, the chromatographic performance remained the same if a NADES solvent was used and so this may only be a problem if the same column is used with different mobile phases, which is often not the case for the majority of routine analysis. When DES and ILs are used as mobile phase additives, their main advantage had been described as the cationic components adsorbing to the exposed silanol groups of the stationary phase, reducing peak tailing, and improving the separation of cationic compounds [35, 50, 51]. Although, not all DES contain a cationic component, in the case of CC-based NADES, this adsorption will likely always occur with any silica-based stationary phase.

It is important to mention that there is currently no "HPLC-grade" NADES so impurities in the NADES that absorb in the UV range or those having fluorescence could appear in the chromatograms when an UV or fluorescence-based detector is hyphenated to the HPLC system. Although it was not tested here, the NADES-based mobile phases would likely be problematic for coupling the HPLC system to detectors where volatilization of the mobile phase is required such as mass spectrometry (MS), evaporative light scattering detector (ELSD), and corona-charged aerosol detector (C-CAD). Precipitation of these impurities or of NADES that have been stored for long periods of time could also be problematic for HPLC equipment. A module to keep NADES under soft agitation together with 50–60 °C of temperature would solve most of the stability problems related to many NADES that would be used as mobile phase major organic components whereas also decreasing their viscosity. As NADES become common solvents, higher purity NADES components can become available making these problems obsolete and allowing the use of all common HPLC detectors that do not require

Table 2 HPLC-EAT scores for an individual separation (first 35 min of the runs) conducted with different mobile phase components

Mobile phase	Safety (*S*) score	Health (*H*) score	Environmental (*E*) score	HPLC-EAT score
LA:Glu:H_2O 5:1:4	27.29	7.66	6.39	41.33
CC:EG 1:2	17.56	9.31	10.43	37.30
CC:EG 1:3	19.49	10.30	11.19	40.98
Ethanol	32.64	3.49	8.38	44.51
Methanol	33.64	7.54	5.61	46.79
Acetonitrile	47.30	18.43	13.39	79.13

volatilization of the mobile phase. No "salting out" effects were observed with the use of these NADES. Between the three NADES examined, CC:EG 1:2 is the most suitable for reducing the amount of organic solvent used in a separation. Although LA:Glu:H_2O 5:1:4 had the most similar retention times to EtOH and ACN, it has a higher wavelength cut-off than the CC-based NADES. CC:EG 1:2 shows a higher elution strength than CC:EG 1:3 making it more suitable for these kinds of separations. The higher elution strength could be related to a denser supramolecular structure which is likely observed with less EG. Additionally, CC:EG 1:2 has the lowest HPLC-EAT score.

Conclusion

From the proof of concept presented here, NADES demonstrated a high potential as green mobile phase components to replace harmful organic solvents in RP-HPLC which have been commonly used for the last 50 years. Considering that the technologies employed in this work were developed to be more compatible with traditional organic solvents than the three NADES tested, the NADES performed well, all giving chromatographic performances in between those observed for ACN and MeOH, which are the two most used organic solvents in HPLC. Although ACN provided the best overall performance for the mixtures tested here, due to the almost infinite possible combinations of DES that could be produced from different compounds, it is possible that other DES could potentially surpass this traditional harmful solvent and that the separation of any specific pair of compounds could be achieved by tailoring DES for this purpose. Moreover, water content and temperature can be used as an extra tool not only for decreasing viscosity and change eluotropic strength of the mobile phase, but additionally for fine tuning selectivity. This could be advantageous as it offers an alternative and green way to improve selectivity other than focusing on the development of new columns. Notwithstanding, the development of appropriate technologies must be considered essential before NADES can be routinely used in HPLC analysis. With these further developments, an automated green extraction method could be possible utilizing NADES as both the extraction solvent and mobile phase similar to what can currently be achieved for organic solvents [52].

Although the selection of NADES tested here was driven by a desire to use the less viscous ones (to be compatible with the maximum backpressure of columns used), a selection guided by environmental parameters would lead to a much greener advantage for NADES compared to traditional organic solvents ACN and MeOH, and even to the greener EtOH. As a result, NADES can be tailored to produce "greener" analyses in addition to tailored selectivity depending on the desired separation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Yuan X, Richter BE, Jiang K, Boniface KJ, Cormier A, Sanders CA, et al. Carbonated water for the separation of carboxylic compounds: a chromatography approach. Green Chem. 2018;20:440–8.
2. Welch CJ, Nowak T, Joyce LA, Regalado EL. Cocktail chromatography: enabling the migration of HPLC to nonlaboratory environments. ACS Sustain Chem Eng. 2015;3:1000–9.
3. Funari CS, Carneiro RL, Cavalheiro AJ, Hilder EF. A trade off between separation, detection and sustainability in liquid chromatographic fingerprinting. J Chromatogr A. 2014;1354:34–42.
4. Fritz R, Ruth W, Kragl U. Assessment of acetone as an alternative to acetonitrile in peptide analysis by liquid chromatography/mass spectrometry. Rapid Commun Mass Spectrom. 2009;23:2139–45.
5. Welch CJ, Wu N, Biba M, Hartman R, Brkovic T, Gong X, et al. Greening analytical chromatography. TrAC Trends Anal Chem. 2010;29:667–80.
6. Płotka J, Tobiszewski M, Sulej AM, Kupska M, Górecki T, Namieśnik J. Green chromatography. J Chromatogr A. 2013;1307:1–20.
7. Koel M. Do we need green analytical chemistry? Green Chem. 2016;18:923–31.
8. Olives AI, González-Ruiz V, Martín MA. Sustainable and ecofriendly alternatives for liquid chromatographic analysis. ACS Sustain Chem Eng. 2017;5:5618–34.
9. Tobiszewski M, Namieśnik J, Pena-Pereira F. Environmental risk-based ranking of solvents using the combination of a multimedia model and multi-criteria decision analysis. Green Chem. 2017;19:1034–42.
10. Prat D, Wells A, Hayler J, Sneddon H, McElroy CR, Abou-Shehada S, et al. CHEM21 selection guide of classical- and less classical-solvents. Green Chem. 2015;18:288–96.
11. Kittell JE Paul P, Arnold D, Neyer D, DeLand P, Rehm J (2008) Micro-scale HPLC generates < 1% of the solvent waste of conventional analytical LC. Paper presented at the the 12th annual green chemistry and engineering conference, Washington DC, USA
12. Armenta S, de la Guardia M. Green chromatography for the analysis of foods of animal origin. TrAC Trends Anal Chem. 2016;80:517–30.
13. Welch CJ, Brkovic T, Schafer W, Gong X. Performance to burn? Re-evaluating the choice of acetonitrile as the platform solvent for analytical HPLC. Green Chem. 2009;11:1232–8.
14. Gaber Y, Tornvall U, Kumar MA, Ali Amin M, Hatti-Kaul R. HPLC-EAT (Environmental Assessment Tool): a tool for profiling safety, health and environmental impacts of liquid chromatography methods. Green Chem. 2011;13:2021–5.
15. Tobiszewski M. Metrics for green analytical chemistry. Anal Methods. 2016;8:2993–9.

16. Funari CS, Carneiro RL, Khandagale MM, Cavalheiro AJ, Hilder EF. Acetone as a greener alternative to acetonitrile in liquid chromatographic fingerprinting. J Sep Sci. 2015;38:1458–65.
17. Lesellier E, West C. The many faces of packed column supercritical fluid chromatography—a critical review. J Chromatogr A. 2015;1382:2–46.
18. Vera CM, Shock D, Dennis GR, Farrell W, Shalliker RA. Comparing the selectivity and chiral separation of D- and L-fluorenylmethyloxycarbonyl chloride protected amino acids in analytical high performance liquid chromatography and supercritical fluid chromatography; evaluating throughput, economic and environmental impact. J Chromatogr A. 2017;1493:10–8.
19. Yang Y. Subcritical water chromatography: a green approach to high-temperature liquid chromatography. J Sep Sci. 2007;30:1131–40.
20. Alghoul ZM, Ogden PB, Dorsey JG. Characterization of the polarity of subcritical water. J Chromatogr A. 2017;1486:42–9.
21. El-Shaheny RN, El-Maghrabey MH, Belal FF. Micellar liquid chromatography from green analysis perspective. Open Chem. 2015;13:877–92.
22. Soares B, Passos H, Freire CSR, Coutinho JAP, Silvestre AJD, Freire MG. Ionic liquids in chromatographic and electrophoretic techniques: toward additional improvements in the separation of natural compounds. Green Chem. 2016;18:4582–604.
23. Han D, Row KH. Recent applications of ionic liquids in separation technology. Molecules. 2010;15:2405–26.
24. Dai Y, van Spronsen J, Witkamp GJ, Verpoorte R, Choi YH. Natural deep eutectic solvents as new potential media for green technology. Anal Chim Acta. 2013;766:61–8.
25. Francisco M, Van Den Bruinhorst A, Kroon MC. Low-transition-temperature mixtures (LTTMs): a new generation of designer solvents. Angew Chem Int Ed. 2013;52:3074–85.
26. Espino M, de los Ángeles Fernández M, FJV G, Silva MF. Natural designer solvents for greening analytical chemistry. TrAC Trends Anal Chem. 2016;76:126–36.
27. Choi YH, van Spronsen J, Dai Y, Verberne M, Hollmann F, Arends IWCE, et al. Are natural deep eutectic solvents the missing link in understanding cellular metabolism and physiology? Plant Physiol. 2011;156:1701–5.
28. Mbous YP, Hayyan M, Wong WF, Looi CY, Hashim MA. Unraveling the cytotoxicity and metabolic pathways of binary natural deep eutectic solvent systems. Sci Rep. 2017;7:41257.
29. Hayyan M, Mbous YP, Looi CY, Wong WF, Hayyan A, Salleh Z, et al. Natural deep eutectic solvents: cytotoxic profile. Springerplus. 2016;5:913.
30. Dai Y, Witkamp GJ, Verpoorte R, Choi YH. Natural deep eutectic solvents as a new extraction media for phenolic metabolites in carthamus tinctorius L. Anal Chem. 2013;85:6272–8.
31. Wei Z, Qi X, Li T, Luo M, Wang W, Zu Y, et al. Application of natural deep eutectic solvents for extraction and determination of phenolics in Cajanus cajan leaves by ultra performance liquid chromatography. Sep Purif Technol. 2015;149:237–44.
32. Radošević K, Ćurko N, Gaurina Srček V, Cvjetko Bubalo M, Tomašević M, Kovačević Ganić K, et al. Natural deep eutectic solvents as beneficial extractants for enhancement of plant extracts bioactivity. LWT-Food Sci Technol. 2016;73:45–51.
33. Bakirtzi C, Triantafyllidou K, Makris DP. Novel lactic acid-based natural deep eutectic solvents: efficiency in the ultrasound-assisted extraction of antioxidant polyphenols from common native Greek medicinal plants. J Appl Res Med Aromat Plants. 2016;3:120–7.
34. Bajkacz S, Adamek J. Evaluation of new natural deep eutectic solvents for the extraction of isoflavones from soy products. Talanta. 2017;168:329–35.
35. Tan T, Zhang M, Wan Y, Qiu H. Utilization of deep eutectic solvents as novel mobile phase additives for improving the separation of bioactive quaternary alkaloids. Talanta. 2016;149:85–90.
36. Li G, Zhu T, Lei Y. Choline chloride-based deep eutectic solvents as additives for optimizing chromatographic behavior of caffeic acid. Korean J Chem Eng. 2015;32:2103–8.
37. Dai Y, Witkamp GJ, Verpoorte R, Choi YH. Tailoring properties of natural deep eutectic solvents with water to facilitate their applications. Food Chem. 2015;187:14–9.
38. De Faria CMQG, Nazaré AC, Petrônio MS, Paracatu LC, Zeraik ML, Regasini LO, et al. Protocatechuic acid alkyl esters: hydrophobicity as a determinant factor for inhibition of NADPH oxidase. Curr Med Chem. 2012;19:4885–93.
39. Snyder LR, Kirkland JJ, Dolan JW. Introduction to modern liquid chromatography. 3rd ed. Hoboken: John Wiley & Sons, Inc.; 2009.
40. Neue UD. Theory of peak capacity in gradient elution. J Chromatogr A. 2005;1079:153–61.
41. Zhang Q, De Oliveira Vigier K, Royer S, Jérôme F. Deep eutectic solvents: syntheses, properties and applications. Chem Soc Rev. 2012;41:7108–46.
42. Prat D, Hayler J, Wells A. A survey of solvent selection guides. Green Chem. 2014;16:4546–51.
43. Martín-Calero A, Pino V, Ayala JH, González V, Afonso AM. Ionic liquids as mobile phase additives in high-performance liquid chromatography with electrochemical detection: application to the determination of heterocyclic aromatic amines in meat-based infant foods. Talanta. 2009;79:590–7.
44. Tobiszewski M, Namieśnik J. Greener organic solvents in analytical chemistry. Curr Opin Green Sustain Chem. 2017;5:1–4.
45. Tobiszewski M, Marć M, Gałuszka A, Namieśnik J. Green chemistry metrics with special reference to green analytical chemistry. Molecules. 2015;20:10928–46.
46. Koller G, Fischer U, Hungerbühler K. Assessing safety, health, and environmental impact early during process development. Ind Eng Chem Res. 2000;39:960–72.
47. Koller G, Fischer U, Hungerbühler K. Assessment of environment-, health- and safety aspects of fine chemical processes during early design phases. Comput Chem Eng. 1999;23:S63–S6.
48. Shaaban H, Górecki T. Current trends in green liquid chromatography for the analysis of pharmaceutically active compounds in the environmental water compartments. Talanta. 2015;132:739–52.
49. Desire CT, Hilder EF, Arrua RD. Monolithic high-performance liquid chromatography columns. In: Encyclopedia of Analytical Chemistry. Hoboken: John Wiley & Sons, Ltd.; 2017. p. 1–37. https://doi.org/10.1002/9780470027318.a9386.
50. Li X, Row KH. Development of deep eutectic solvents applied in extraction and separation. J Sep Sci. 2016;39:3505–20.
51. García-Alvarez-Coque MC, Ruiz-Angel MJ, Berthod A, Carda-Broch S. On the use of ionic liquids as mobile phase additives in high-performance liquid chromatography. A review. Anal Chim Acta. 2015;883:1–21.
52. Ferreira VG, Leme GM, Cavalheiro AJ, Funari CS. Online extraction coupled to liquid chromatography analysis (OLE-LC): eliminating traditional sample preparation steps in the investigation of solid complex matrices. Anal Chem. 2016;88:8421–7.