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Countercurrent assisted quantitative recovery of metabolites from plant-associated natural deep eutectic solvents

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ABSTRACT

NAtural Deep Eutectic Solvents (NADES) are chemically simple but physiologically important plant constituents that exhibit unique solubilizing properties of other metabolites, including bioactive constituents. The high polarity of NADES introduces a challenge in the ability of conventional solid-support based chromatography to recover potential bioactive metabolites. This complicates the systematic explanation of the NADES' functions in botanical extracts. The present study utilizes countercurrent separation (CCS) methodology to overcome the recovery challenge. To demonstrate its feasibility, Glucose-Choline chloride-Water (GCWat, 2:5:5, mole/mole) served as a model NADES, and four widely used marker flavonoids with different polarities (rutin, quercetin, kaempferol, and daidzein) were chosen as model target analytes. In order to prepare GCWat with high consistency, a water drying study was performed. The unique capabilities of the recently introduced CherryOne system, offering volumetric phase metering, were used to monitor the CCS operations. The collected fractions were analyzed using UHPLC and NMR/quantitative NMR. CCS was able to recover the analytes from the NADES matrix with quantitative recoveries of 95.7%, 94.6%, 97.0%, and 96.7% for rutin, quercetin, kaempferol, and daidzein respectively. The CCS strategy enables recovery of target metabolites from NADES-containing crude extracts as well as from other chemical mixtures, and moreover offers a means of using NADES as environmentally friendly extraction solvents.

1. Introduction

In 2011, NAtural Deep Eutectic Solvents (NADES) were identified from plants as a special form of mixtures of solids that remain in the liquid state at and below ambient temperature, and show distinctively different physiochemical characteristics from both water and lipids [1]. The NADES components were recognized as being "primary" metabolites [1,2], such as amino acids, organic acids, and sugars.

Habitually, natural products have been divided into "primary" (biochemical housekeeping) and "secondary" (frequently bioactive) metabolites. However, in the absence of a theoretical basis, this metabolite classification has been shown to be inadequate [3]. The historic partitioning of compounds into the two groups also illustrates a general but potentially deceptive relationship with polarity: "primary" metabolites are mostly highly polar, water soluble compounds, whereas the "secondary" metabolites tend to be more lipophilic. Meanwhile, numerous exceptions apply to this general rule, e.g., when quaternary alkaloids are classified as being "secondary" or fatty acids as being "primary". However, this grouping still reflects the tendency of polar (primary) metabolites to represent the major portion of the currently defined metabolome in plants, but also other organisms. Conversely, the structurally more complex "secondary" metabolites typically represent the smaller, more lipophilic portion, granted varying degrees of overlap.

Typical "primary" plant metabolites are sugars (e.g., glucose, fructose, mannose, sucrose), organic salts (e.g., choline chloride, betaine), organic acids (e.g., lactic acid, citric acid, malic acid), and amino acids (e.g., proline, serine, alanine). Interestingly, these types of metabolites have also been found to be NADES constituents. Despite their high polarity, NADES exhibit an unexpected solubilizing ability for relatively lipophilic compounds [2] and can even stabilize bioactive metabolites [4]. As NADES components, "primary" metabolites may have important (non-housekeeping) functions in botanical extracts, including (over-)additive biological effects that are often referred to as "synergistic". This hypothesis is nurtured by the observation that

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dietary supplements and traditional medical formulae continue to be administered in the form of the crude extracts, which often are rich in NADES, and show distinctly different pharmacodynamic properties relative to purified materials. However, as the "primary" metabolites are usually considered to have no intrinsic bioactivity, or even to represent "nonsense" compounds, their role in this context has remained essentially unstudied. Therefore, the recognition of NADES opens new perspectives in natural product research.

One important prerequisite for studying the function of the "primary", potentially NADES-forming metabolome in botanical extracts is the ability to separate cleanly the polar "primary" from the potentially bioactive "secondary" metabolites. Another complication relates to the polar nature of NADES: many of the "primary" metabolites show unfavorable chromatographic characteristics in solid-support liquid chromatographic methods, in particular in preparative procedures that are key to the characterization of botanical active principles. One part of the challenge is associated with the inherently low vapor pressure of NADES, which paired with their high viscosity makes recovery of analytes from NADES media difficult when using conventional liquid chromatography (LC) [5,6]. To overcome this deficiency, an alternative chromatography needs to be developed.

Countercurrent separation (CCS) is a liquid-only form of LC [7,8]. This technology is orthogonal to solid phase-based LC, can avoid sample loss resulting from degradation or absorption on a solid support, and thus, is characterized by high recovery and reproducibility [9,10]. At the same time, as the stationary phase in CCS is a liquid, its separation and resolution essentially depend on the partition coefficient (K) value of each analyte, representing their relative distribution between the stationary and mobile phases of a given solvent system [11]. Unlike most natural products, polar NADES components are able to form strong hydrogen bonds [12]. Accordingly, NADES tend to be soluble in polar solvents such as water and MeOH, while being insoluble in non-polar solvents such as EtOAc and hexane [13]. Therefore, the NADES components will prefer to stay in an aqueous phase and may be eluted at, or very near the front or tail of the elution in a CCS, far away from the usual K value sweet spot range from 0.25 to 16 [14]. Following this discussion, if a given target analyte can be delivered into the sweet spot range of K values, a clean separation of the analyte from a NADES-analyte matrix is likely achievable. This was one of the key hypotheses of the present study.

The second set of rationales related to the choice of a model NADES system and target analytes for proof-of-concept purposes. Considering our research focus on botanicals for women's health, certain flavonoids have been widely considered as bioactive constituents and, thus, were identified as potential model compounds. On one hand, some function as important bioactive ingredients, e.g., the isoflavone, daidzein, from red clover extract, exhibits estrogenic activity [15–17]. On the other hand, several have recently been identified as potential invalid metabolic panaceas (IMPs) [18], compounds that have led to wasted effort and resources. Accordingly, many of them are representatives of the top-ranking IMP candidates, and can even undermine the drug discovery and discovery of bioactive botanical markers. The glucoside, rutin, and the aglycones, quercetin and kaempferol, are representatives of the top-ranking IMP candidates [18]. Accordingly, these three flavonoids as well as the isoflavone, daidzein, were selected as model metabolites for the present study. One goal was to show that CCS is able to not only enrich bioactive constituents for subsequent screening of potential leads, but also to knock out the IMPs quantitatively, avoiding their otherwise unavoidable or unwanted interference in screening bioassays. Equally important, the advancement of a clean cut between "secondary" and "primary" metabolites would enable the investigation of the understudied "primary" metabolite function. For model NADES selection, the system of Glucose-Choline chloride-Water (GCWat, 2:5:5, mole/mole) was chosen due to its abundance in plants and the fact that it exhibits a relatively high solubility for flavonoids including the relatively lipophilic aglycones and the glycoside, rutin [2]. Each individual flavonoid was soluble in GCWat, and four individual GCWat-analyte solutions (rutin at 20 mg/mL, quercetin at 18 mg/mL, kaempferol and daidzein at 4 mg/mL), were used to demonstrate the feasibility of CCS-assisted recovery of analytes from the NADES matrix.

2. Experimental

2.1. Materials

Choline chloride, D-(+)-glucose, rutin, UHPLC grade solvents, and DMSO-d_6 (99.9 at.% D) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Quercetin, kaempferol, and daidzein were obtained from UIC/NIH Center for Botanical Dietary Supplements Research. The analytical grade solvents were purchased from Pharmco-AAPER (Crookfield, CT, USA) and redistilled before use.

2.2. Preparation of natural deep eutectic solvent and related solutions

Glucose-Choline chloride-Water (GCWat, 2:5:5, mole/mole) was prepared as follows: glucose (0.01 mole) and choline chloride (0.025 mole) were dissolved in distilled water (2 mL) and treated in an FS140 ultrasonic bath (Fisher Scientific, Loughborough, UK) until particles were completely dissolved. Then, the solution was placed in a centrifugal vacuum evaporation system (Thermo Scientific, Waltham, MA, USA) to remove water for 14.4 h. The evaporation system consisted of an SC250 Express SpeedVac Concentrator (37 °C and 4.7 Torr), a RVT4104 Refrigerated Vapor Trap (4 L and −104 °C), and an OFP 400 Vacuum Pump. After 14.4 h of vacuum centrifugal evaporation, the desired NADES, GCWat was obtained.

NADES-analyte solutions were prepared as follows: the analytes were mixed with the appropriate volume of GCWat in a vial. The vial was then placed into an ISOTEMP 110 water bath (Fisher Scientific, Loughborough, UK) at 37 °C for 24 h. The following NADES-analyte solutions were produced: rutin (20 mg/mL), quercetin (18 mg/mL), kaempferol (4 mg/mL), and daidzein (4 mg/mL).

2.3. Countercurrent chromatography procedures

2.3.1. Biphasic solvent system selection

The TLC-based Generally Useful Estimate of Solvent System (GUESS) method was used for the selection of the biphasic solvent systems. Rutin, quercetin, kaempferol, and daidzein dissolved in MeOH (1 mg/mL) were spotted individually on silica gel TLC plates (Macherey-Nagel, USA). All TLC plates were developed with the organic phase of each candidate solvent system [14,19], and the resulting chromatograms were screened for ones in which the Rf value of each test sample was close to 0.5. All solvent systems used and the results obtained are listed in Table 1.

2.3.2. Countercurrent separation (CCS)

CCS was performed as previously described [20] in a TBE-20 A HSCCC instrument (16 mL, Tauto Biotech, China). The NADES-analyte solution (100 μL) was diluted with both upper and lower phase (200 μL of each), then loaded into the sample loop (2 mL). Several different solvent systems were used in this study, for details see Table 1 and Section 3.2. In order to optimize recovery, the dipped part of the tube attached to the flush out port and the vial were washed three times with lower phase (300 μL/each) and also loaded into the sample loop. Reversed phase

Table 1
The screened CCS solvent systems and their related TLC results.

Rutin			Quercetin	Kaempferol	Daidzein
EBuWat	Rf values	HEMWat	Rf values		
5:5:10	0.59	3:7:5:5	0.63	0.73	0.62
6:4:10	0.53	4:6:5:5	0.36	0.59	0.37
7:3:10	0.41	5:5:5:5	0.20	0.37	0.21