



Broad range chemical profiling of natural deep eutectic solvent extracts using a high performance thin layer chromatography–based method



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ABSTRACT

Natural deep eutectic solvents (NADES) made mainly with abundant primary metabolites are being increasingly applied in green chemistry. The advantages of NADES as green solvents have led to their use in novel green products for the food, cosmetics and pharma markets. However, one of the main difficulties encountered in the development of novel products and their quality control arises from their low vapour pressure and high viscosity. These features create the need for the development of new analytical methods suited to this type of sample. In this study, such a method was developed and applied to analyse the efficiency of a diverse set of NADES for the extraction of compounds of interest from two model plants, *Ginkgo biloba* and *Panax ginseng*. The method uses high-performance thin-layer chromatography (HPTLC) coupled with multivariate data analysis (MVDA). It was successfully applied to the comparative qualitative and quantitative analysis of very chemically diverse metabolites (e.g., phenolics, terpenoids, phenolic acids and saponins) that are present in the extracts obtained from the plants using six different NADES. The composition of each NADES was a combination of two or three compounds mixed in defined molar ratios; malic acid-choline chloride (1:1), malic acid-glucose (1:1), choline chloride-glucose (5:2), malic acid-proline (1:1), glucose-fructose-sucrose (1:1:1) and glycerol-proline-sucrose (9:4:1). Of these mixtures, malic acid-choline chloride (1:1) and glycerol-proline-sucrose (1:1:1) for *G. biloba* leaves, and malic acid-choline chloride (1:1) and malic acid-glucose (1:1) for *P. ginseng* leaves and stems showed the highest yields of the target compounds. Interestingly, none of the NADES extracted ginkgolic acids as much as the conventional organic solvents. As these compounds are considered to be toxic, the fact that these NADES produce virtually ginkgolic acid-free extracts is extremely useful. The effect of adding different volumes of water to the most efficient NADES was also evaluated and the results revealed that there is a great influence exerted by the water content, with maximum yields of ginkgolides, phenolics and ginsenosides being obtained with approximately 20% water (w/w).

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1. Introduction

Natural products (NPs) are undoubtedly the most plentiful source of new bioactive compounds and play an important role in our daily lives, being used for their medicinal, nutritional and cosmetic properties, and in industrial applications. However,

extraction from their natural sources is generally a complex process, consisting of several steps that involve the use of large volumes of organic solvents. Unfortunately, most of these solvents are banned in the use of products for human consumption due to their toxicity or are extremely restricted. In general, these solvents are also highly volatile, posing as hazards to the environment. For these reasons, the choice of suitable solvents is very limited [1].

The green extraction of NPs can be achieved by using innovative extraction techniques and/or sustainable alternatives to conventional solvents. Great improvements have been accomplished with the use of non-conventional techniques such as ultrasound-assisted extraction, microwave-assisted extraction,

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membrane-mediated extraction, and micro-extraction [2]. Some of these techniques even allow for solvent-free extraction, including microwave hydro-diffusion and gravity, enzyme-assisted extraction [3] and thermal desorption systems. However, for large-scale extraction, the use of solvents is practically unavoidable hence it is necessary to find alternatives to the conventional organic solvents and/or mineral acids. Amongst the green options that have appeared so far, the most popular are super- or subcritical fluids and ionic liquids. Supercritical fluid extraction (SFE), in particular with supercritical carbon dioxide (SC-CO₂), has been used extensively for years to extract many natural bioactive compounds or eliminate toxic compounds from a variety of materials [4–6]. However, despite its environmental benefits, the efficiency of CO₂ for the extraction of polar/hydrophilic compounds and macromolecules is limited and many bioactive or nutritional compounds, such as phenolic glycosides or alkaloids, are not extractable due to their poor solubility in CO₂. Other alternatives in the green list include conventional solvents such as water, certain agro-solvents (e.g., ethanol and glycerol) and surfactant aqueous solutions [7,8].

Another type of novel alternative solvents that have gained considerable attention within the past few years are ionic liquids (ILs). ILs are salts that consist of certain combinations of cations and anions with melting points that are below 100 °C. These liquids possess many attractive physicochemical properties such as low volatility, some electrolytic conductivity and tunable viscosity and miscibility, making them promising substitutes for organic solvents in numerous processes like biocatalytic processes, extractions, catalysis, and electrochemistry [9–11]. However, their toxicity, poor biodegradability and the high cost of synthesising their major components cause major hindrance for their widespread application as extraction solvents.

Another option, deep eutectic solvents (DES), are mixtures of specific organic compounds (not necessarily ionic) that have a much lower melting point than either of the individual components and, similarly to ILs, are liquids at ambient temperatures [12]. DES exhibit similar physicochemical properties as commonly used ILs (e.g., high density and viscosity) but have the advantages of being significantly cheaper, easier to prepare and less impactful on the environment. In addition to this, they can be tailored to be target-specific. These unique properties allow for a wide range of applications of DES in fields such as extraction, catalysis, materials chemistry, organic synthesis, metal processing, and electrochemistry [13,14], as well as in the extraction of DNA and as a Media for enzymatic reactions [15,16].

A number of obstacles remain in the application of DES, particularly in those made with synthetic components in which toxicity is an issue. Searching for alternatives, Choi and his co-workers have explored more than 100 combinations of DES using only common metabolites that are abundant in plants [17]. They named these combinations; 'natural deep eutectic solvents' (NADES) [17]. These liquids are bio-based DES which are composed of two or more compounds that are generally functional primary metabolites, i.e., organic acids, sugars, (poly)alcohols, amines and amino acids [17,18]. Apart from sharing the favorable characteristics of ILs and DES as described previously, NADES have additional advantages of being composed of naturally-occurring compounds, being much more sustainable and posing practically no environmental hazards. This highlights the great potential of NADES for its application in various areas, namely in the NP field as an extraction solvent. Currently, the most studied application of NADES as solvents for NPs is the extraction of different phenolic compounds from plant material such as *Carthamus tinctorius* flowers [19], *Sophora japonica* flowers [20], *Cajanus cajan* leaves [21], *Catharanthus roseus* flowers [22], grape peel [23], and anthocyanins from wine lees [24]. They have also been applied for the extraction of ginsenosides

from *Panax ginseng* [25] and food contaminants [26]. Recently, the first commercial NADES plant extracts were launched as cosmetic ingredients and in the coming years a number of novel NADES-based products can be expected in the food, cosmetic and pharma markets.

Given the increasing amount of NADES applications, the methodological problems related to the analysis of NADES extracts must be addressed. These problems are related mainly to the negligible volatility of NADES that makes the recovery of compounds from NADES extracts or the elimination of interfering NADES components very difficult. So far, most analyses of NADES extracts have been focused on phenolics that pose few methodological challenges given their highly-absorbing chromophores that allow direct UV-spectrophotometric analysis with few to no sample clean-up procedures. Different chromatographic methods have been used to identify and quantify individual components, including the determination of the anthocyanin content in flower petal extracts from *C. roseus* [19] and flavonoids in flower extracts from *S. japonica* via HPLC-DAD, using anti-solvent strategies for recovery [20]. Alongside chromophore compounds, volatiles have been relatively easily analysed by GC following simple liquid-liquid extraction from a choline chloride and 4-chlorophenol (1:2) NADES [27]. However, none of these methods can be applied to the full range of very chemically diverse compounds that are present in NADES extracts from biological materials.

Within the past five decades, thin layer chromatography (TLC) has advanced greatly from a simple planar chromatographic technique for major qualitative profiling into a multi-target quantitative analytical tool with many applications in the field of medicinal plants. The main disadvantages of TLC, such as its low resolution and poor quantitative performance, have been considerably improved by the optimisation of the conventional TLC set-up. Nowadays, the basic steps of TLC-based methods, i.e., sample application, chromatographic development and detection, can be automated and computer-controlled. Other undesirable characteristics, such as low efficiency, have been improved with the use of high-performance thin-layer chromatography (HPTLC) plates, which use stationary phases with smaller particle sizes (5–6 µm) and high-resolution sorbents with improved silica particles and chemically-modified phases. These modern systems feature high reproducibility in retention, detection and quantitative analysis. For further statistical evaluation, electronic images of the chromatograms can be generated and used for multivariate data analysis. As for the identification of compounds, notable progress has been made in the direct online coupling of HPTLC with mass spectrometry (MS) [28–31] which introduces new opportunities for the application of planar chromatography in metabolomics.

Combining all of these improvements with the advantages of multi-sample analysis in a single run and the option for selective detection of a wide range of chemically diverse compounds makes HPTLC a useful tool for the qualitative and quantitative analysis of NADES extracts from natural products. Furthermore, the application of multivariate image analysis to HPTLC chromatograms allows for the extraction of incalculably more information than that made available by simple visual inspection [31].

The aim of the current study was to develop a suitable analytical protocol for the HPTLC analysis of NADES plant extracts and then to implement it to investigate the application of diverse NADES (including a variation in water content) with the extraction of three chemically different groups of active compounds in *Ginkgo biloba* (i.e., ginkgolides, phenolics and ginkgolic acids) [33] and of ginsenosides from *Panax ginseng* [34–37]. The influence of adjustments to the water content on the extraction ability and efficacy of the most efficient NADES was also studied due to its known impact on their physicochemical properties.

2. Materials and methods

2.1. Plant material

Ginkgo biloba leaves were collected in Seoul, Republic of Korea in 2012. *Panax ginseng* leaves and stems were kindly provided by Fytogoras (Leiden, the Netherlands). Samples used for this study were identified by one of the authors, Dr. Y. H. Choi and a voucher sample was deposited in the Natural Products Laboratory, Institute of Biology, Leiden University. The dry plant material was powdered in a blender with liquid nitrogen.

2.2. Chemicals and reagents

Bilobalide, ginkgolides (A, B and C), ginkgolic acids (C13:0, C15:1 and C17:1) and ginsenosides (Rb1, Rb2, Rb3, Re, Rg1, Rg2 and Rg3) were purchased from Biopurify Phytochemicals (Chengdu, China). Rutin, chlorogenic acid and quercetin were purchased from Sigma (St. Louis, MO, USA). Methanol, ethanol, chloroform, acetone, ethyl acetate and toluene of analytical grade were purchased from Sigma. Acetic anhydride, acetic acid and formic acid were obtained from Sigma. Milli-Q water was used. Polyethylene glycol 400 was purchased from Alfa Aesar (Kandel, Germany). All of the NADES components, i.e., choline chloride ($\geq 98.0\%$), glycerol ($\geq 99.5\%$), L-proline ($\geq 99.0\%$), D-fructose ($\geq 99.0\%$), D-glucose ($\geq 99.5\%$), malic acid ($\geq 99.0\%$), and sucrose ($\geq 99.5\%$) were obtained from Sigma. Sodium acetate and 2-aminoethyl diphenylborinate were purchased from Sigma. Solid phase extraction (SPE) cartridges (OASIS HLB 3cc) were purchased from Waters (Milford, MA, USA). Silica gel 60 F254 HPTLC plates were purchased from Merck (Darmstadt, Germany).

2.3. NADES preparation

The NADES employed in this study were combinations of fixed molar ratios as follows; malic acid-choline chloride (1:1, N1), malic acid-glucose (1:1, N2), choline chloride-glucose (5:2, N3), malic acid-proline (1:1, N4), glucose-fructose-sucrose (1:1:1, N5) and glycerol-proline-sucrose (9:4:1, N6) (Table S1). The first five NADES were prepared by stirring mixtures of their components at 50 °C until a clear liquid was formed [19], whereas glycerol-proline-sucrose (9:4:1) was prepared using the freeze-drying method [25]. All of the prepared NADES were mixed with 10% (w/w) water. The two most efficient NADES for the extraction of each plant were found to be malic acid-choline chloride (1:1) and glycerol-proline-sucrose (9:4:1) for *G. biloba*, and malic acid-choline chloride (1:1) and malic acid-glucose (1:1) for *P. ginseng*. These NADES were selected to further study the effect of adding varying amounts of water (0%, 10%, 20%, 30% and 40%, w/w) on their extraction properties.

2.4. Preparation of reference compound solutions for HPTLC analysis

All reference solutions were prepared separately in methanol in the following concentrations: bilobalide and ginkgolides B and C (250 $\mu\text{g/mL}$); ginkgolide A and C13:0, C15:1 and C17:1 ginkgolic acids (125 $\mu\text{g/mL}$); rutin (1.8 $\mu\text{g/mL}$); chlorogenic acid and quercetin (4 $\mu\text{g/mL}$); ginsenosides Rb1, Rb2, Rb3, Re, Rg1, Rg2, and Rg3 (30 $\mu\text{g/mL}$).

2.5. Preparation of extracts and sample solutions for HPTLC analysis

Powdered plant material (200 mg) was mixed with 4 mL methanol or NADES in a 15-mL centrifugation tube. After vortex-

ing for 1 min, the mixture was placed into a water bath at 40 °C for 1 h and then ultrasonicated at room temperature for 30 min. The mixture was then centrifuged at 13,000 rpm for 20 min. An aliquot of 1 mL of the supernatant was used for the HPTLC analysis following pre-treatment. All extracts were prepared by triplicate. Extracts prepared with methanol were used to compare the yield of the diverse NADES extracts.

2.6. Recovery of samples from NADES

The removal of NADES from the mixture was conducted with solid-phase extraction (SPE) using HLB cartridges [25]. Briefly, a cartridge was placed in a vacuum manifold and equilibrated with 5 mL of ethanol, followed by 5 mL of water. After loading the extract solution (1 mL), the cartridge was subsequently rinsed with 6 mL of water twice and then eluted with 6 mL of ethanol. The ethanol eluate was dried and re-dissolved in 1 mL of methanol for HPTLC analysis.

2.7. General HPTLC analysis

Reference and sample solutions (100 μL) were spotted by triplicate on HPTLC Si 60 F254, 20 $\times$ 10 cm (Merck) plates as 7 mm bands, under a stream of nitrogen, using the CAMAG Automatic TLC sampler (ATS 4) (CAMAG, Muttens, Switzerland) with a 100 μL Hamilton syringe. The CAMAG auto-sample system is controlled by Wincats software. All plates were prepared similarly, spotting the methanol extracts in the first three lanes, the reference solutions in the next three and then the NADES extracts.

2.8. Analysis of NADES extracts with 10% water (w/w)

The layout of the tracks on the HPTLC plates varied in each case. For the ginkgolides in *G. biloba*, a total of 13 tracks were used, including triplicates of the methanol extract, four references and six different NADES extracts. Bands were applied at a distance of 10 mm from the bottom of the plate and 16 mm from the left and the right edges. For the determination of phenolics and ginkgolic acids in *G. biloba*, the number of tracks per plate was 12, i.e., triplicates of methanol extract, three references and six different NADES extracts. Bands were applied at a distance of 10 mm from the bottom of the plate and 20 mm from the left and the right edges. For ginsenosides in *P. ginseng* leaves and stems, the number of tracks per plate was 17, i.e., duplicates of methanol extract of leaves and stems respectively, one reference and six different NADES extracts of leaves and stems respectively. Bands were applied at a distance of 10 mm from the bottom of the plate and 18 mm from the left and the right edges.

2.9. The use of NADES with different water contents

The extracts obtained from *G. biloba* material with the most efficient NADES (N1 and N6, see above) with different added water contents were then compared with each other and against the methanol extracts. In the case of ginkgolides, this resulted in 17 tracks per plate with 16 used for the phenolics and ginkgolic acids, i.e., triplicates of the methanol extract, three references, and the two NADES with five different water contents. Bands were applied at a distance of 10 mm from the bottom of the plate and 16 mm from the left and the right edges. With the NADES extraction of ginsenosides from *P. ginseng* leaves and stems, the effect of added water contents was studied using the most efficient NADES in this case, i.e., N1 and N2. There were 14 tracks on each plate including triplicates of the methanol extract, one reference, and the N1 and N2 extracts with five different water contents. Bands were applied at a distance of 10 mm from the bottom of the plate and 18 mm from the

left and the right edges. The HPTLC conditions used for the analysis were those described for *G. biloba* and *P. ginseng* in the application notes of CAMAG laboratory (F-16A, F-16B, F-16C) [38] and the *Chinese Pharmacopeia* [39], respectively (Table S2). Derivatisation reagents were applied with an auto-spraying instrument (Derivatizer, CAMAG).

2.10. Determination of ginkgolides in *Ginkgo biloba* leaves

Prior to sample spotting, the plates were immersed in an ethanolic solution of sodium acetate (8 g NaOAc in 200 mL of 80% aqueous ethanol) for 2 s, allowed to dry in the hood for 5 min at room temperature and then activated at 90 °C for 30 min as indicated in the application notes of CAMAG laboratory (F-16A) [38]. Volumes of 25 µL of methanol extracts and reference solutions, and 80 µL of NADES extract solutions were spotted onto the HPTLC plate as described. The plate was developed in a saturated chamber with a mobile phase of toluene-ethyl acetate-acetone-methanol (20:10:10:1.2, v/v/v/v). After drying the plates to remove the mobile phase, they were evenly sprayed with acetic anhydride and heated at 180 °C for 10 min. Densitometric scanning was performed at UV 366 nm. Bilobalide and ginkgolides A, B and C were used as reference compounds.

2.11. Determination of phenolics in *Ginkgo biloba* leaves

A volume of 25 µL of each reference solution, methanol extract, and NADES extracts was applied onto the HPTLC silica plate and developed with a mobile phase consisting of ethyl acetate-acetic acid-formic acid-water (100:11:11:27, v/v/v/v) in a chamber saturated for 20 min before use, according to the application note of CAMAG laboratory (F-16B) [38]. For the derivatisation, the plate was heated at 100 °C for 3 min on a TLC Plate Heater (CAMAG), then dipped first in Natural Products reagent (1% 2-aminoethyl diphenylborinate in methanol), dried with cold air, and then subsequently dipped in PEG reagent (5% polyethylene glycol 400 in dichloromethane). Densitometric scanning was performed at UV 366 nm after derivatisation. Rutin, chlorogenic acid and quercetin were used as reference compounds.

2.12. Determination of ginkgolic acids in *Ginkgo biloba* leaves

A volume of 25 µL each of reference solutions of ginkgolic acids (C13:0, C15:1 and C17:1), the methanol extract and the NADES extracts was spotted separately onto the plate and developed with a mobile phase consisting of toluene-ethyl acetate-acetic acid (8:2:0.2, v/v/v) in a saturated chamber as indicated in the F-16-C of the application notes of CAMAG laboratory [38]. The plate was dried with a stream of cold air and scanned at UV 366 nm.

2.13. Determination of ginsenosides in *Panax ginseng* leaves and stems

The plate was developed with chloroform-ethyl acetate-methanol-water (15:40:22:10) in a chamber saturated for 20 min [39]. Ginsenosides were detected by spraying the plate with freshly prepared anisaldehyde-sulfuric acid reagent and heating on a plate heater at 105 °C for 5 min. Images were captured under white light after derivatisation. Ginsenosides Rb1, Rb2, Rb3, Re, Rg1, Rg2 and Rg3 were used as standards.

2.14. Image processing and multivariate analysis

The HPTLC chromatograms were processed using rTLC software [32] which converts the HPTLC images into a numerical data matrix which is then integrated. This process generated digital data of 50

sequential 0.02 bins over the full retention factors (R_f) range for each track. An R_f of 0.02 was selected as the bin size because it represents the individual band in a single bin but avoids the R_f drift which may result from batch-to-batch variation factors. To reduce the variations between the replicates that were performed at different times, the intensity recorded for each R_f value was normalised with respect to the methanol extract (reference extract sample) in each plate. These 0.02 R_f bins were subjected to multivariate data analysis. Principal component analysis (PCA) and orthogonal partial least square (OPLS) were performed with the SIMCA-P software (v. 14.1, Umetrics, Umeå, Sweden). The unit variance (UV) scaling method was used both for PCA analysis and OPLS modelling.

3. Results and discussion

This study was performed using six types of NADES to extract four groups of chemically diverse bioactive compounds (phenolics, terpenoids and phenolic acids from *G. biloba* leaves, and ginsenosides from *P. ginseng* leaves and stems). The NADES have been grouped into five typical types according to their components, i.e., NADES composed of acids and bases (N1), acids and sugars (N2), bases and sugars (N3), amino acid and acids (N4), and sugar mixtures (N5). In addition to these typical NADES, glycerol-proline-sucrose (9:4:1, N6) was selected because in previous work performed by Jeong and her co-workers, it was reported that ginsenosides were well-extracted from *P. ginseng* roots by the NADES [25]. To reduce the high viscosity of the NADES as an extraction solvent, 10% of water (w/w) was added to each NADES, as suggested in a previously published paper [18].

Similarly to conventional synthetic DES or ILs, NADES extracts have virtually zero vapour pressure which means that the solvent cannot be removed by evaporation as is generally the case when organic solvents are used for extraction. The removal of the extraction solvent is generally necessary when analysing an extract, concentrating the low-level compounds or avoiding its interference with the analysis. In the case of NADES, two approaches have been proposed, namely liquid-liquid partitioning [27] and solid phase extraction (SPE) with diverse sorbents [20,25]. However, neither method completely removed all of the NADES. Liquid-liquid partitioning is only possible with non-polar solvents such as *n*-hexane, dichloromethane or chloroform because most NADES components are soluble in polar or mid-polar solvents, making it difficult to separate them from the extracted polar secondary metabolites with similar polarities. On the other hand, SPE has proved to be quite efficient in purifying secondary metabolites, though NADES residues may still cause problems in various analytical methods.

So, there is a clear need for efficient methods that can be applied to the vast range of chemically diverse compounds found in NADES extracts. Thin layer chromatography has a long tradition in NP analysis and, in the past decade, has evolved into a highly improved technique known as HPTLC [40]. This technique meets all of the mentioned requirements to a great extent. In particular, the possibility of the simultaneous analysis of several samples on a single plate and the possibility of a preparative work by mass and/or NMR spectroscopy constitute a great advantage over other chromatographic methods [41,42].

To develop HPTLC protocols for the analysis of NADES extracts, we used two well-known medicinal plants as models, *G. biloba* and *P. ginseng*. The plant material was extracted with six different types of NADES. These extracts were then analysed by HPTLC for the presence of four different types of compounds; phenolics, terpenes, lactones and alkylphenols in *G. biloba* leaves, and triterpene saponins in *P. ginseng* leaves and stems.

The NADES extracts were first analysed directly, without any pre-purification steps, to evaluate the interference of the NADES in

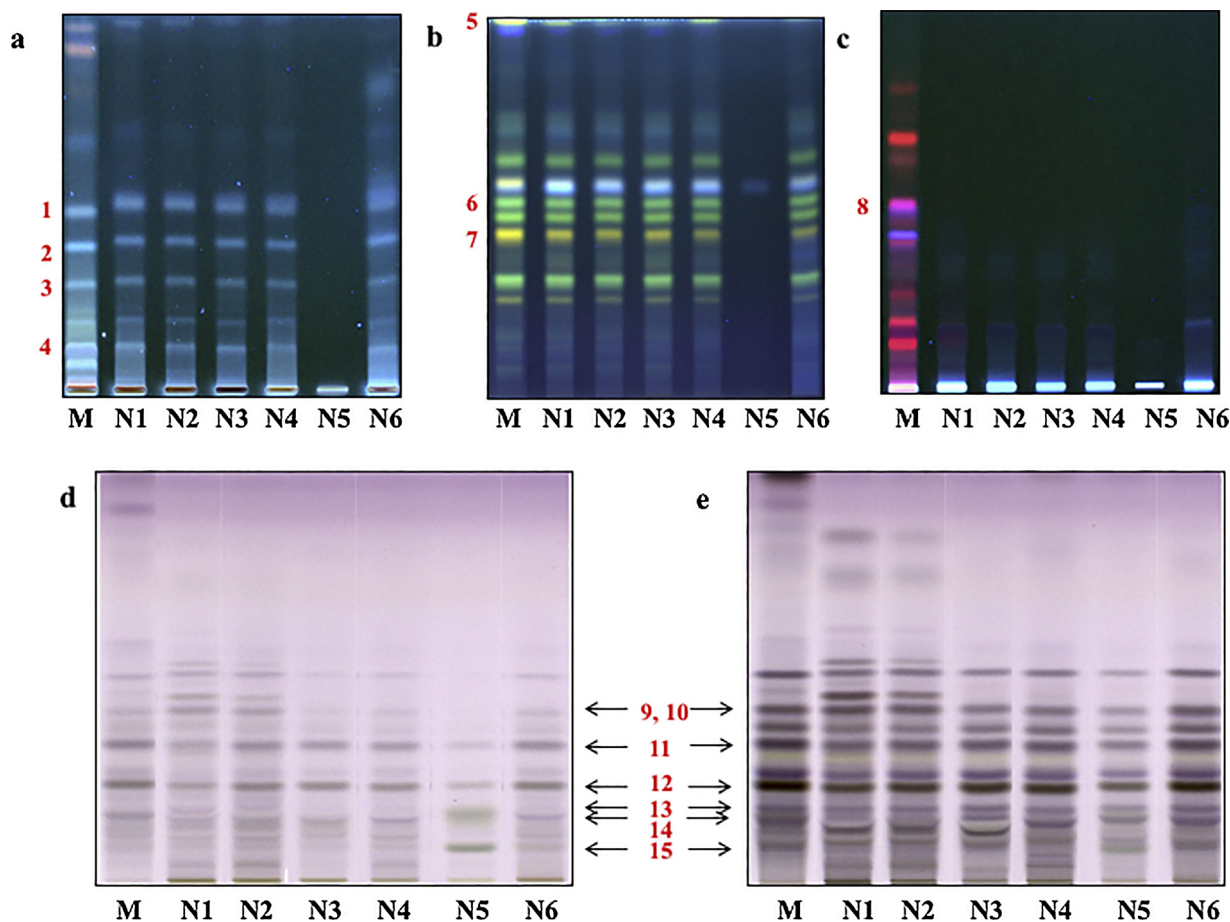


Fig. 1. High-performance thin-layer chromatographs (HPTLC) of methanol extracts and six natural deep eutectic solvent (NADES) extracts with 10% (w/w) water for ginkgolides (a), ginkgo phenolics (b), and ginkgolic acids (c) of *Ginkgo biloba* leaves, and ginsenosides of *Panax ginseng* stems (d) and leaves (e). Ginkgolides were analysed after derivatisation with acetic anhydride at 366 nm using plates that were impregnated with an ethanolic solution of sodium acetate (see details in M&M). Ginkgo phenolics were detected after derivatisation with the natural products reagent (NPR) and PEG 4000 at 366 nm. Ginsenosides were visualised under white light after treatment with the anisaldehyde-sulfuric acid reagent. The detailed HPTLC conditions were provided in the experimental section. M: methanol extract, N1–N6: NADES extracts. N1: malic acid-choline chloride (1:1), N2: malic acid-glucose (1:1), N3: choline chloride-glucose (5:2), N4: malic acid-proline (1:1, molar ratio), N5: glucose-fructose-sucrose (1:1:1), N6: glycerol-proline-sucrose (9:4:1). Reference compounds: 1: bilobalide, 2: ginkgolide A, 3: ginkgolide B, 4: ginkgolide C, 5: quercetin, 6: chlorogenic acid, 7: rutin, 8: ginkgolic acids (C13:0, C15:1 and C17:1), 9: ginsenoside Rg3, 10: ginsenoside Rg2, 11: ginsenoside Rg1, 12: ginsenoside Re, 13: ginsenoside Rb3, 14: ginsenoside Rb2, 15: ginsenoside Rb1.

the HPTLC methods for the four different groups of NPs. The presence of NADES caused severe tailing of spots in all of the systems. It was clearly necessary to perform some sample clean-up procedures, hence SPE was selected as the method of choice for this. Because of their ability to bind a wide range of secondary metabolites, including glycosides, Oasis HLB cartridges were tested for the purification of ginsenosides from the NADES extracts composed of glycerol, proline and sucrose (9:4:1) [25]. The NADES extract was introduced onto the cartridge and the NADES components were removed by an initial elution with water, after which the compounds of interest were eluted with ethanol. The quality of the HPTLC separation improved as tailing completely disappeared.

The NADES extracts of *G. biloba* leaves and *P. ginseng* leaves and stems were all treated in the same way and then analysed by HPTLC. Fig. 1 shows the four groups of metabolites that each can be well visualised in the different HPTLC chromatograms, clearly showing its power to detect a wide range of chemically diverse groups of metabolites [43]. The *P. ginseng* saponins without UV-chromophores were able to be visualised at 254 nm and 366 nm after treatment with the anisaldehyde-sulfuric acid derivatisation reagent (Fig. 1d, e).

Visual examination of the HPTLC chromatograms showed that the extraction efficiency of all NADES employed in this study,

except for the all-sugar NADES N5, was similar to that of methanol for ginkgolides and phenolics from *G. biloba* and for ginsenosides in *P. ginseng* leaves and stems (Fig. 1). Ginkgolic acids were not significantly extracted by any of the NADES. In general, plant aliphatic phenols like ginkgolic acids have very low polarity, which makes them difficult to be dissolved in polar solvents. Most NADES are categorised as polar solvents and could not adequately extract the non-polar ginkgolic acids.

In the cases of ginkgolides and ginsenosides, the NADES extracts showed fewer bands than the methanol extracts, but all of the main compounds (bilobalide, 3 ginkgolides, and 7 ginsenosides) in the NADES extracts were still present in similar concentrations to that of the methanol extracts with an exception for the sugar mixture (N5) (Fig. 1a, d, e). In the case of *G. biloba* phenolics, NADES extracts displayed more bands than the methanol extracts, for example, within the 0.35–0.38 R_f range (Fig. 1b). The most striking feature, however, is low extraction yield of the ginkgolic acids in all tested NADES revealing clearly different extraction profiles for the NADES and methanol (Fig. 1c). Ginkgolic acids are considered to be toxic and the presence of these compounds is unwanted in *G. biloba* extracts that are used for human consumption, so the extraction of the leaves with NADES could result in high quality Ginkgo preparations with very low ginkgolic acid content.

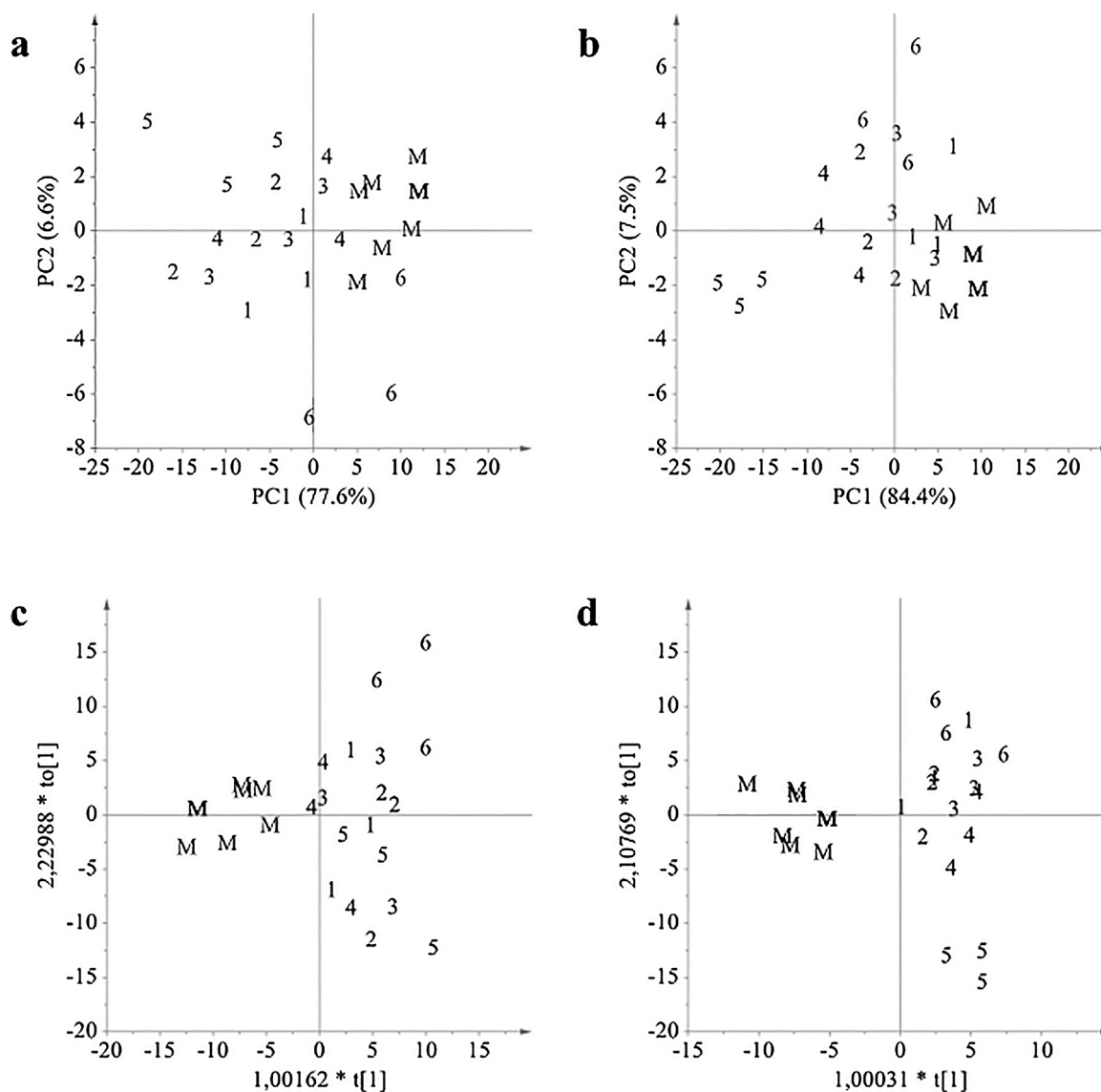


Fig. 2. Score plot of principal component analysis (PCA) of natural deep eutectic solvent (NADES) extracts and methanol extracts of ginkgolides (a) and ginkgo phenolics (b) in *Ginkgo biloba* leaves, and score plots of orthogonal partial least square discriminant analysis of ginkgolides (c) and ginkgo phenolics (d). M: methanol extract. 1–6: NADES extracts. 1: malic acid-choline chloride (1:1, molar ratio), 2: NADES of malic acid-glucose (1:1), 3: choline chloride-glucose (5:2), 4: malic acid-proline (1:1), 5: glucose-fructose-sucrose (1:1:1), 6: glycerol-proline-sucrose (9:4:1).

The results obtained in this study highlight once more the great potential of NADES as a green alternative solvent for the extraction of phenolics. This high extraction power of NADES for phenolics may be related to H-bonding interactions between the functional groups of the components (e.g., hydroxyl and carboxyl groups) and the hydroxyl groups in phenolics. We have reported the observation of H-bonding interactions between quercetin and NADES in previous studies [44].

Chromatographic profiles provide basic information about specific groups of compounds but, most importantly, can characterise the chemical composition of a sample in a holistic way. In fact, that is the paradigm of metabolomics; aiming at the unbiased analysis of all of the metabolites within an organism. In order to fully take advantage of all of the information provided by this profiling method, it is necessary to use biometric methods such as multivariate data analysis (MVDA) and multivariate image analysis to be able to identify the similarities and differences between the measured profiles and then combine these with other observations, including

the metabolic changes triggered by diseases or related to resistance against herbivores.

All chromatographic profiling methods require the normalisation and alignment of signals prior to MVDA. For the normalisation, three control samples (in this study a methanol extract was used) were spotted alongside the other samples on each plate. The intensity at each R_f value of the samples were normalised to a methanol extract in order to minimise the variation of replicates on different plates. This normalisation improved the quality of the MVDA data quality (Fig. S1). The recently-developed open-source software, rTLC, was used for alignment which offers a standardised procedure for image processing and the visualisation tools that are required to compare HPTLC fingerprints via different pattern recognition and prediction techniques [32]. The processed data were further analysed by PCA and OPLS.

The PCA data of ginkgolides and ginkgo phenolics in *G. biloba* leaves is shown in Fig. 2. No further analysis of the ginkgolic acids in NADES extracts of *G. biloba* leaves was performed due to their low

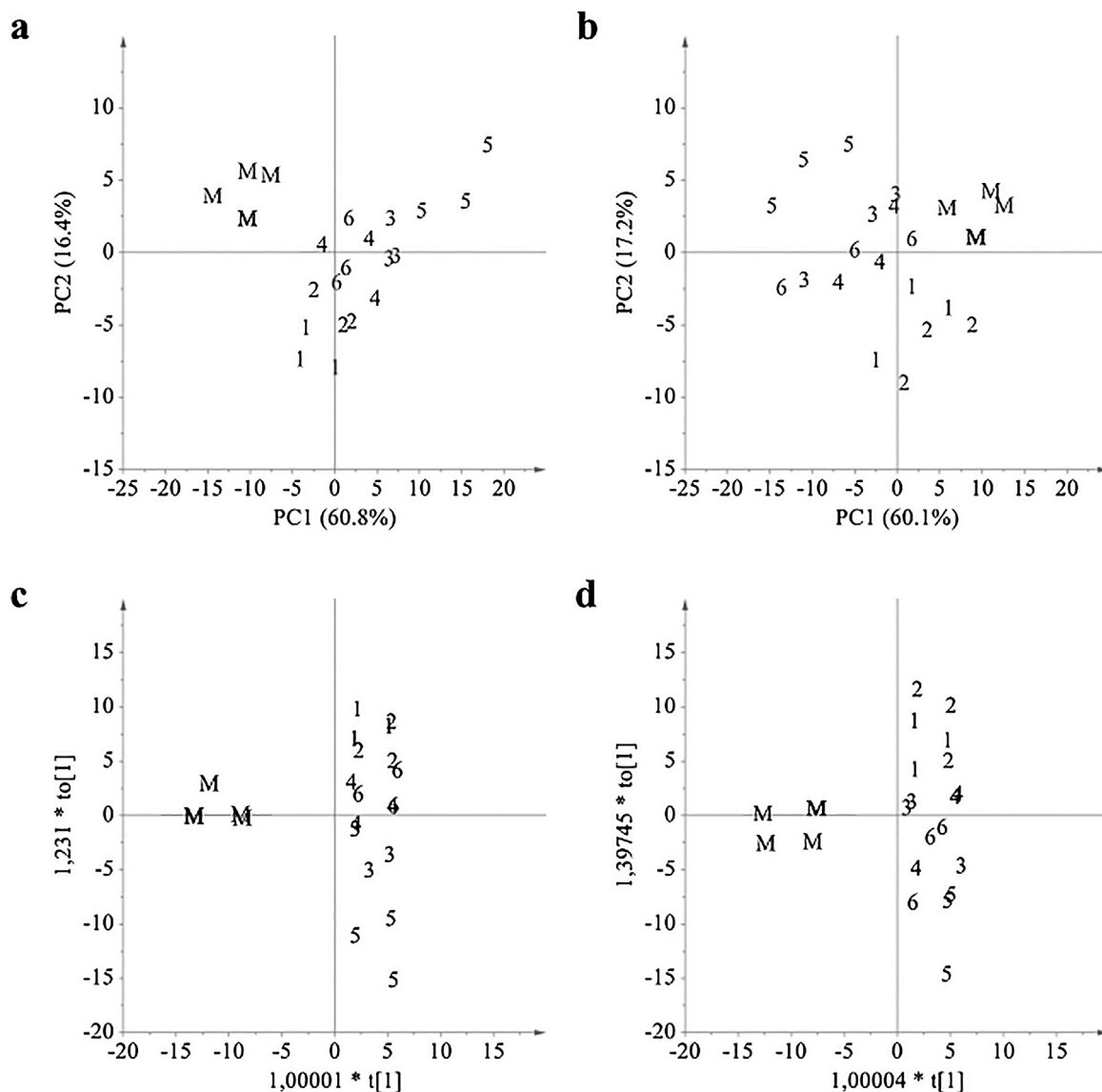


Fig. 3. Score plots of principal component analysis (PCA) of data corresponding to ginsenosides in *Panax ginseng* leaves (a) and stems (b) obtained from HPTLC chromatograms using PC1 and PC2 comparing various natural deep eutectic solvents (NADES) extracts and methanol extracts. Orthogonal partial least square-discriminant analysis (OPLS-DA) score plots of ginsenosides in *P. ginseng* leaves (c) and stems (d). The numbering of the extracts is the same as in Fig. 2.

yield as shown in Fig. 1c. In the PCA score plot of *G. biloba* samples, NADES extracts were clearly distinct from the methanol extract but there was no significant difference amongst the NADES solvents. If any, in the case of phenolics (Fig. 2b), malic acid-choline chloride (N1) and glycerol-proline-sucrose (N6) appeared to be closer to the methanol extracts than the other NADES extracts. A supervised MVDA, OPLS-DA, was employed to obtain a more detailed comparison between both the methanol and NADES extracts, including all of the six tested NADES. This showed a clear separation between methanol and NADES extracts (Fig. 2d). For the identification of the contributing metabolites, an S plot was used and it revealed that methanol extracted higher amounts of most metabolites. All of the tested ginkgolides, bilobalide (R_f 0.447), ginkgolides A (R_f 0.361), B (R_f 0.266) and C (R_f 0.114) were extracted less efficiently with NADES (Fig. S2a). The HPTLC analysis of phenolics in *G. biloba* leaves also acknowledged that methanol was more efficient than NADES, for example, for chlorogenic acid (R_f 0.52), rutin (R_f 0.352) and quercetin (R_f 0.99) (Fig. S2b).

In the case of *P. ginseng* leaves and stems, there were differences amongst the extraction profiles obtained with methanol, but also amongst the NADES extracts (Fig. 3a, b). The first cluster consisted of the methanol extracts, the second of malic acid-choline chloride (N1) and malic acid-glucose (N2), and the third grouped together the three remaining NADES, choline chloride-glucose (N3), malic acid-proline (N4) and glycerol-proline-sucrose (N6), implying a similarity in their chemical profiles. Extracts made with glucose-fructose-sucrose (N5) formed a fourth cluster (Fig. 3a and b). To compare the NADES and methanol extracts, OPLS-DA was applied to the *P. ginseng* samples (Fig. 3c and d) and the results showed that all of the seven analysed ginsenosides were more efficiently extracted from both *P. ginseng* leaves and stems with NADES (Fig. S3).

Apart from their chemical composition, another factor that has a great influence on the physicochemical properties of NADES is their water content [45]. One of the positive effects of increasing the water content is a decrease in their viscosity; one of the features

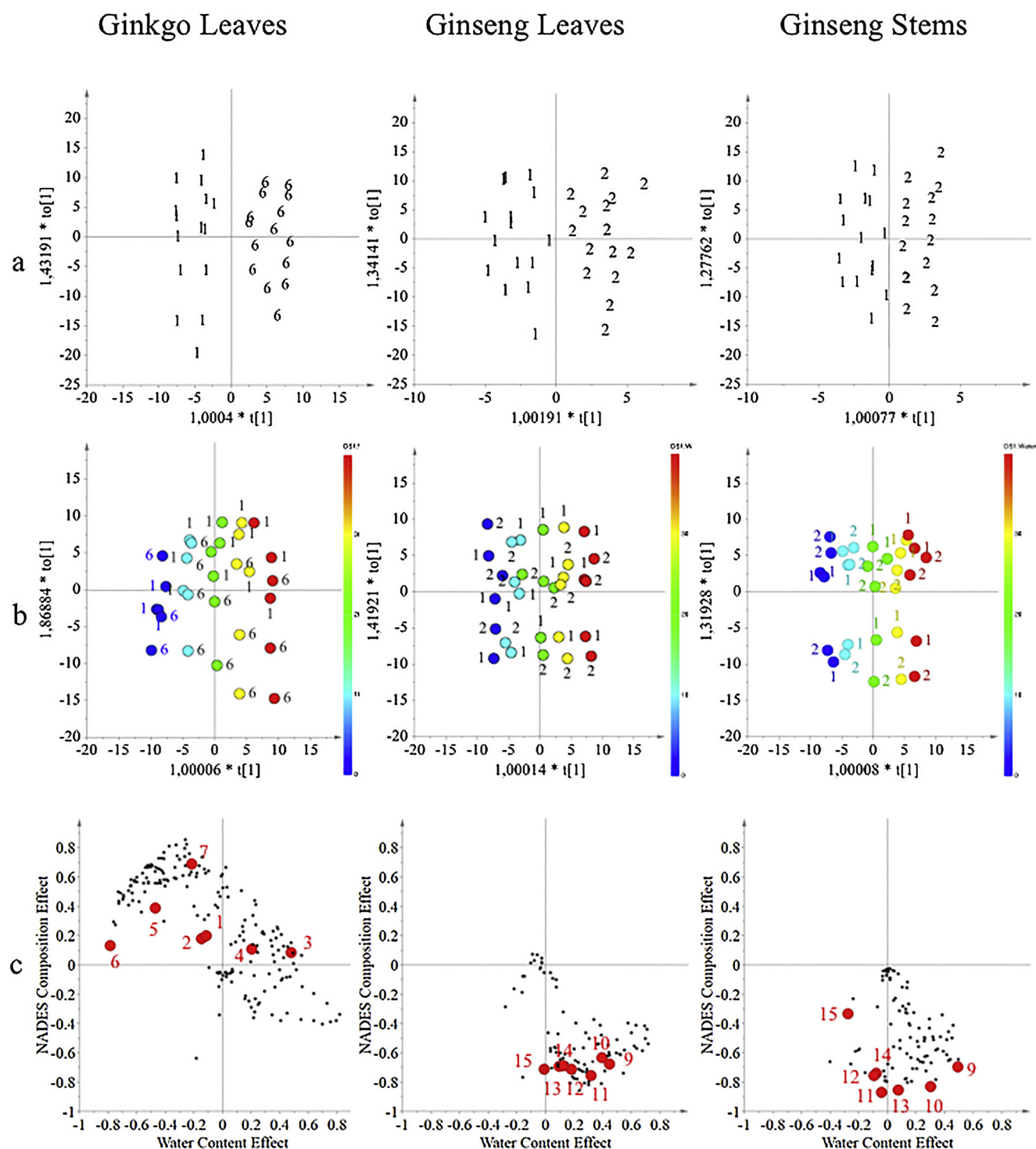


Fig. 4. Orthogonal partial least square-discriminant analysis (OPLS-DA) score plots of different natural deep eutectic solvents (NADES) extracts (a), OPLS score plots of various water contents (0–40%, w/w) (b) and shared-and-unique-structures (SUS)-plots (c) of ginkgolides and ginkgo phenolics in *Ginkgo biloba* leaves, and ginsenosides in *Panax ginseng* leaves and stems. SUS plots correlate the two OPLS-DA models with the X-axis of NADES composition and OPLS of water contents as Y-axis. The numbering of the extracts in Fig. 4a and b is the same as in Fig. 2. The numbering of the identified compounds is the same as in Fig. 1.

that hinders their use as extraction solvents. To evaluate the effect of the water ratio, different amounts of water was added (0–40%, w/w) to the two NADES that had the highest extraction yields for each plant in the first experiment. The extracts were analysed using HPTLC-MVDA.

The content of ginkgolides and phenolics in *G. biloba* extracts prepared with malic acid-choline chloride (1:1) (N1) and glycerol-

proline-sucrose (9:4:1) (N6) with varying water ratios were compared. The data obtained from the HPLC chromatograms (X-data) were combined with the water content (Y-data) to evaluate the effect of the water percentage (w/w) on the tested NADES. Two different OPLS models with the water content or composition of NADES as Y-data were set up (Fig. 4a, b). In the score plot of the OPLS modeling, the two selected NADES extracts were clearly sep-

arated from each other (Fig. 4a). From the results, it was clear that apart from the different chemical compositions of N1 and N6, the water content also had a large effect on the extraction profiles. These two OPLS models (NADES composition of N1 and N6, and water content) were integrated by a shared-and-unique-structures (SUS)-plot, in which diagonally-aligned metabolites are of equal importance and shared by the two models, and the main factor influencing extraction yield of each metabolite can be deduced (Fig. 4c). In the SUS-plot, the effect of individual factors (X-axis for NADES chemical compositions and Y-axis for water content) were easily distinguished for each metabolite. As observed, the two metabolites that are most affected by the NADES composition changes were ginkgolide B and chlorogenic acid, though very differently, i.e., glycerol-proline-sucrose (N6) extracted the highest amount of ginkgolide B but the least amount of chlorogenic acid. The water content greatly influenced NADES extraction yields as seen on the Y-axis in the SUS-plot (Fig. 4c). This was particularly noticeable in the case of rutin.

Panax ginseng leaves and stems were extracted with the two most efficient NADES, malic acid-choline chloride (N1) and malic acid-glucose (N2), with varying volumes of added water and then analyzed by OPLS and SUS-plot, similarly to the *G. biloba* samples. Interestingly, as seen in Fig. 4, the yield of the seven ginsenosides were influenced in a different way, even though their structural differences are minimal. The highest yield of ginsenosides Rg3 and Rg2 in both *P. ginseng* leaves and stems were obtained with malic acid-glucose (N2) whilst malic acid-choline chloride (N1) yielded the most ginsenoside Rb1 from *P. ginseng* stems (Fig. 4c). All seven ginsenosides of *P. ginseng* leaves and six ginsenosides of *P. ginseng* stems (except for ginsenoside Rb1) were best extracted with NADES with the highest added water content (Fig. 4c).

4. Conclusion

To develop a reliable analytical method for NADES extracts, a HPTLC-based method was employed. This method was tested on two well-known medicinal plants and proved to be able to deliver reproducible chemical profiles from the NADES extracts. The results verified that the yield of bioactive compounds obtained with six different types of NADES is similar to that of methanol in all cases with only one exception. The application of multivariate analysis revealed, however, some clear differences in extraction selectivity amongst the different NADES. The addition of water to the NADES had a large effect on the efficiency of their extraction for the selected compounds, increasing their yield in general. Maximum amounts of ginkgolides, phenolics and ginsenosides were obtained with an addition of approximately 20% water to the NADES. It is worth noting that the most striking difference between the methanol and NADES extracts was the significant lack in ginkgolic acids in the NADES extracts. This is very promising for further studies, since it suggests potential for obtaining practically ginkgolic acid-free preparations for pharmaceutical use.

All of the results obtained in this study show NADES to be promising extraction solvents. A deeper knowledge of the theoretical basis for the extraction mechanism of the NADES and their interaction with solutes would greatly facilitate the development of future applications. Aside from this, the HPTLC-analytical method described here will be a useful tool for this process.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.chroma.2017.12.009>

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