Isolation of DNA from Plant Tissues using a Miniaturized Matrix Solid-phase Dispersion Approach Featuring Ionic Liquid and Magnetic Ionic Liquid Solvents

Shashini De Silva,[1] Iran Ocaña-Rios,[1] Cecilia Cagliero,[2] Morgan R. Gostel,[3]
Gabriel Johnson,[4] and Jared L. Anderson[1,*]

[1] Department of Chemistry, Iowa State University, Ames, Iowa 50011, USA.
[2] Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, I-10125, Turin, Italy.
[3] Botanical Research Institute of Texas, Fort Worth, Texas 76107-3400, USA.
[4] Smithsonian Institution, Suitland, Maryland 20746, USA.

Abstract

The isolation of high-quality plant genomic DNA is a major prerequisite in many plant biomolecular analyses involving nucleic acid amplification. Conventional plant cell lysis and DNA extraction methods involve lengthy sample preparation procedures that often require large amounts of sample and chemicals, high temperatures and multiple liquid transfer steps which can introduce challenges for high throughput applications. In this study, a simple, rapid, miniaturized ionic liquid (IL)-based extraction method was developed for the isolation of genomic DNA from milligram fragments of *Arabidopsis thaliana* plant tissue. This method is based on a modification of vortex-assisted matrix solid-phase dispersion (VA-MSPD) in which the trihexyl(tetradecyl)phosphonium bis(trifluoromethylsulfonyl)imide ($[\mathrm{P}_{6,6,6,14}^{+}][\mathrm{NTf}_2^{-}]$) IL or trihexyl(tetradecyl)phosphonium tris(hexafluoroacetylaceto)nickelate(II) ($[\mathrm{P}_{6,6,6,14}^{+}][\mathrm{Ni(hfacac)}_3^{-}]$) magnetic IL (MIL) was directly applied to treated plant tissue (~1.5 mg) and dispersed in an agate mortar to facilitate plant cell lysis and DNA extraction, followed by recovery of the mixture with a qPCR compatible co-solvent. This study represents the first approach to use ILs and MILs in a MSPD procedure to facilitate plant cell lysis and DNA extraction. The DNA-enriched IL- and MIL-cosolvent mixtures were directly integrated into the qPCR buffer without inhibiting the reaction while also circumventing the need for additional purification steps prior to DNA amplification. Under optimum conditions, the IL and MIL yielded 2.87±0.28 and 1.97±0.59 ng of DNA/mg of plant tissue, respectively. Furthermore, the mild extraction conditions used in the method enabled plant DNA in IL- and MIL-cosolvent mixtures to be preserved from degradation at room temperature.

Keywords: Plant DNA isolation; Ionic liquids; Magnetic ionic liquids; *Arabidopsis thaliana;* Matrix solid phase dispersion; qPCR

* Corresponding author:
Jared L. Anderson
Department of Chemistry
Iowa State University
Ames, IA 50011
Tel.: +1 515-294-8356
E-mail address: andersoj@iastate.edu

1. Introduction

Many types of plant biomolecular analyses including genotyping [1], sequencing [2], mutation screening [3], and plant pathogen detection [4] require amplification of nucleic acids by polymerase chain reaction (PCR), considered to be the gold standard approach. The first step in such applications is isolation of nucleic acids from the plant matrix, a challenging task that involves tedious sample preparation procedures. Plants offer more complexity for cell lysis and DNA extraction mainly due to the presence of rigid cell walls and varying levels of secondary metabolites. Contaminants present in plant tissues, if not properly removed from DNA, can ultimately result in PCR inhibition. Among the methods that have attempted to resolve these challenges are the widely used cetyltrimethylammonium bromide (CTAB) [5] or sodium dodecylsulfate (SDS) plant cell lysis protocols [6]. These traditional methods generally use surfactants and heat to lyse the plant cell walls, resulting in release of the cellular components to a buffer followed by multiple centrifugation steps to remove the insoluble particulate matter. Additional purification of DNA from soluble proteins and polysaccharide contaminants is carried out by phenol-chloroform extraction, followed by ethanol or isopropanol precipitation. Although these methods give rise to high yields of DNA, major disadvantages include lengthy procedures, multiple liquid handling and transfer steps, the use of harmful chemicals such as phenol and chloroform and the requirement of large amounts of sample [7]. To accelerate extractions, commercial solid-phase extraction kits with silica-based spin columns have been designed. These kits utilize lysis buffers containing either CTAB or SDS, binding buffers comprised of chaotropic salts to facilitate adsorption of DNA to the silica sorbent and wash buffers containing organic solvents to elute and purify the DNA [8]. Conventional plant cell lysis and DNA extraction methods require time-consuming sample preparation steps that often involve or generate numerous quantitative polymerase chain reaction (qPCR) inhibitors, which can limit their applicability in high throughput applications. To address these challenges, consolidated approaches that combine rapid lysis and DNA extraction steps to shorten analyses while also eliminating unwanted contamination are needed.

Recently, ionic liquids (ILs) and magnetic ionic liquids (MILs) have been explored as novel solvents in the extraction of DNA from complex biological matrices. ILs are organic molten salts featuring melting temperatures at or below 100 °C. They possess desirable physicochemical properties such as negligible vapor pressures, high ionic conductivity, and high chemical stability

[9–11]. These properties, coupled with high tunability of cation and anion chemical structures, make ILs attractive solvents in a wide variety of bioanalytical applications [12]. Recent studies have demonstrated that ILs are capable of lysing cells from different biological materials such as plants, meat, viruses, or bacteria while also extracting DNA within very short periods of time [13–16]. MILs are a subclass of ILs that are produced by incorporating a paramagnetic component in the cation and/or anion [17–20]. MILs combine the advantageous properties of ILs with strong magnetic susceptibility permitting the rapid recovery of analyte-enriched MIL from aqueous solutions with the aid of an external magnet [21,22]. Due to their excellent extraction capabilities, MILs have been used for the extraction of nucleic acids from whole blood cells, as well as bacterial and plant cell lysates [23–26]. Some of these studies have demonstrated interactions that facilitate DNA extraction by these solvents include electrostatic interactions between the cation of the solvents and the negatively charged phosphate backbone of the DNA as well as hydrophobic interactions between alkyl chains of the solvents and the bases of DNA [23,24].

Marengo et al. first used MILs to extract genomic DNA from a plant cell lysate using dispersive liquid-liquid microextraction (DLLME) [25]. In this approach, a SDS based lysis step was performed at 100 °C to generate the plant lysate followed by extraction of DNA using MILs. Recently, Emaus et al. reported a one-step plant cell lysis and DNA extraction method incorporating hydrophobic ILs and MILs that circumvented the need for a lengthy temperature-controlled lysis step [27]. This study demonstrated that ILs and MILs alone are capable of lysing plant cells and extracting DNA from intact plant tissue and the amount of DNA extracted increases with longer times and higher temperatures. However, a drawback of using hydrophobic ILs and MILs in the direct extraction of genomic DNA from solid matrices, such as plants, is the high viscosity which interferes with the precision of measuring extraction efficiencies. Lukacs et al. has demonstrated that the diffusion of DNA fragments in the cytoplasm is impeded with increasing DNA size [28]. Therefore, the extraction of genomic DNA from solid matrices to highly viscous solvents provides a significant challenge as it is desired to attain highly quantitative and repeatable results when sampling fragments of plant tissue from the same specimen. Moreover, conventional methods are not amenable to miniaturization due to sample loss during multiple transfer and centrifugation steps, especially when minute amounts of plant samples are used.

To overcome the aforementioned challenges, optimization of IL/MIL-based nucleic acid isolation methods should emphasize the following features: (1) development of a miniaturized method that incorporates very small amounts of plant sample to improve sample utility and reduce consumption of solvents and sample preparation time; (2) blending of the plant tissue sample and IL/MIL to completely disrupt the sample and maximize interactions with the solvent; (3) reduction of IL/MIL viscosity by using components that are qPCR compatible; (4) rapid and efficient extraction of DNA from very small plant samples at room temperature to avoid incubation at elevated temperatures; (5) preservation of DNA from degradation and denaturation.

Matrix solid-phase dispersion (MSPD) is an ideal alternative to conventional sample preparation methods and is able to fulfil a number of the aforementioned optimization features. MSPD is a simple, efficient and versatile technique that was developed for the extraction of analytes from solid, semi-solid and/or highly viscous biological samples [29–31]. A typical MSPD procedure involves mechanical blending and dispersion of the sample with a suitable sorbent material to obtain a homogenous mixture, followed by packing the blended sample into a solid phase extraction (SPE) cartridge and elution of the target analytes with an appropriate solvent [29,32]. Several modifications to the classical MSPD procedure have been developed to make the procedure simple or to increase extraction yield [33]. Some modified procedures include ultrasonic-assisted MSPD (UA-MSPD) [34], vortex-assisted MSPD (VA-MSPD) [35], magnetically-assisted MSPD (MA-MSPD) [36], and Soxhlet-assisted MSPD (SA-MSPD) [37]. Among these modified MSPD procedures, VA-MSPD substitutes the column elution step of classical MSPD with vortex to minimize solvent use and extraction time [33]. VA-MSPD involves blending of the sample and sorbent mixture and transferring the mixture into a centrifuge tube, followed by addition of the extraction solvent and a brief vortex step. Finally the sample is centrifuged and the supernatant analyzed [33]. Recent advances in MSPD-based applications have been made by employing new dispersant materials such as carbon nanotubes (CNTs) [38], graphene [39], molecularly imprinted polymers [40] and ionic liquids [41,42]. Another interesting feature of MSPD is the ability to miniaturize the entire process which aids in improving sample utility while minimizing sample loss, consumption of solvents and sample preparation time [33,43]. Additionally, the mild extraction conditions used in MSPD prevent analytes from degradation and denaturation [30].

In this study, a microscale sample preparation method was developed through the integration of ILs and MILs into a miniaturized VA-MSPD procedure to extract genomic DNA from plants. The treated plant tissue was ground with either IL or MIL to facilitate simultaneous and homogenous plant cell disruption and extraction of DNA into the solvent followed by recovery of the mixture with a co-solvent. The recovered plant extract was briefly vortexed and separated by centrifugation. A number of experimental parameters including sample dehydration approach, type of tissue, mass of plant tissue, type and volume of extraction solvent as well as volume of co-solvent were assessed and optimized. The sample preparation approach was coupled with qPCR to enable highly sensitive quantification of genomic DNA from milligram fragments of *Arabidopsis thaliana* plant tissue. An additional purification step prior to the amplification step was not required due to compatibility of the solvents with qPCR. DNA stored in IL- and MIL-cosolvent mixtures was capable of being amplified after 21 days of storage.

2. Materials and methods

2.1 Reagents, Materials and Equipment

Nickel(II) chloride (98%), 1,1,1,5,5,5-hexafluoroacetylacetone (99%) and ammonium hydroxide (28–30% solution in water) were purchased from Acros Organics (Morris Plains, NJ, USA). Cobalt(II) chloride (97%), lithium bis[(trifluoromethyl)sulfonyl]imide ($[Li^+][NTf_2^-]$), methanol (99.7%) and hexane (≥98.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trihexyl(tetradecyl)phosphonium chloride $[P_{6,6,6,14}^+][Cl^-]$) (97.7%) was purchased from Strem Chemicals (Newburyport, MA, USA). Dimethyl formamide (99.8%), dimethyl sulfoxide (DMSO) (≥99.7%), optically clear PCR caps, tube strips and isopropanol (99.9%) were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Anhydrous diethyl ether (99.0%) was purchased from Avantor Performance Materials Inc. (Center Valley, PA, USA). All primers were acquired from Integrated DNA Technologies (Coralville, IA, USA). SsoAdvanced Universal SYBR Green Supermix purchased from Bio-Rad Laboratories (Hercules, CA, USA) was used for the qPCR assays. SYBR Green I (10,000x) was purchased from Life Technologies (Carlsbad, CA, USA). All aqueous solutions were prepared using 18.4 MΩ cm deionized water obtained from a MilIipore Milli-Q water purification system (Bedford, MA, USA). An Elechomes UH401 food dehydrator (Elechomes, China) was used for removal of residual solvent in the leaf dehydration experiments. An Eppendorf I24 incubator shaker (Eppendorf, Hamburg, Germany) was used as an incubator for extraction experiments. An Agate mortar (50 mm O.D. x 43 mm I.D. x 12 mm depth) with a pestle was acquired from MSE supplies (Tucson, AZ, USA). A household microwave oven

(Kenmore, Model 405.73099310, 900W) was used for experiments involving microwave treatment of the samples.

2.2 MIL and IL synthesis

The IL and MILs explored in this study were synthesized and characterized based on previously reported procedures [17]. Their chemical structures are shown in Table 1. The synthesized MILs and IL were stored in a desiccator when not in use.

2.3 Plant growth conditions

Wild-type *Arabidopsis thaliana* (L.) Heynh, Col 0 seeds purchased from Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA) were grown at 25 °C under ambient conditions. Plant leaves were collected approximately 2 weeks after germination using sterilized scissors. All leaves were air-dried at room temperature until a constant weight was reached, unless otherwise specified.

2.4 Preparation of DNA standard and qPCR amplification

Genomic DNA required for the preparation of standard solutions was isolated using a NucleoSpin Plant II commercial kit (Macherey–Nagel, Düren, Germany) following the manufacturer's specifications. The concentration of DNA isolated by the kit was determined by fluorometric detection using Qubit 4.0 fluorometer (ThermoFisher Scientific, Waltham, MA, USA) with the double-stranded DNA (dsDNA) high sensitivity assay.

Quantification of DNA extracted by the ILs and MILs was performed using qPCR by amplification of the internal transcriber spacer (ITS) region of the plant genome that is conserved amongst plants [44]. The forward and reverse primers for qPCR amplification of the ITS region were 5'-GCA TCG ATG AAG AAC GCA GC-3'and 5'-TCC TCC GCT TAT TGA TAT GC-3', respectively [44]. The qPCR buffer used for reactions containing 0.5 µL of the $[P_{6,6,6,14}^{+}][NTf_2]$ IL-DMSO-water, $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$ MIL-DMSO, or $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$ MIL-DMSO mixtures required 1× SsoAdvanced Universal SYBR Green Supermix, 200 nM of each ITS primer and an additional 1× SYBR green I for a total volume of 20 µL. All reactions were performed on a Bio-Rad CFX96 Touch Real-time PCR thermocycler (Hercules, CA, USA) according to the following thermocycling protocol: initial denaturation step of 10 min at 95 °C and 40 cycles comprised of a 15 s denaturation step at 95 °C and a 45 s annealing step at 65 °C followed by an optical detection step. Melt curve analysis was carried out after qPCR amplification starting at 65 °C for 5 s and increasing to 95 °C in 0.5 °C increments. The cycle of quantification (Cq) values obtained by the qPCR experiments were used to assess the amount of amplifiable DNA. To determine the mass of genomic DNA extracted by $[P_{6,6,6,14}^{+}][NTf_2^{-}]$ IL and $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$ MIL, a 5-point calibration curve was constructed by plotting the Cq (cycle of quantification) value against the log of mass of DNA per reaction. The qPCR efficiency and linearity were calculated for all calibration curves to assess any possible inhibition that may hinder amplification. All qPCR experiments were carried out in triplicate, unless specified otherwise.

2.5 Extraction procedures

2.5.1 IL-based direct solid-liquid extraction

The general IL-based direct solid-liquid extraction procedure used in this study is shown in Figure 1. A 10 µL volume of $[P_{6,6,6,14}^{+}][NTf_2^{-}]$ IL, $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$ MIL or $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$ MIL was added to 1.0 mg of air-dried *A. thaliana* plant material placed within a qPCR tube and incubated at room temperature for 1 h. The DNA-enriched IL or MIL was recovered and 0.5 µL of the recovered solution was added to the qPCR assay for quantification. All extractions were conducted in triplicate. The effects of incubation time, temperature, solvent volume, and sample pretreatment were examined in this study.

2.5.2 IL-based direct solid-liquid extraction using a co-solvent

To mitigate viscosity issues of the IL and the MIL, DMSO and DMF were explored as co-solvents. The fresh and air-dried tissues were cut into 4 symmetrical parts and the cut leaf fragments were weighed and immersed in 15 µL of the IL or MIL within a qPCR tube and incubated at room temperature for 1 h. After incubation, the co-solvent was added to the IL-plant mixture and vortexed for 30 s to homogenize the solution. A 0.5 µL volume of the mixture was added to the qPCR assay for quantification. The effects of different volume of DMSO and DMF on qPCR were also explored.

2.5.3 Modification of IL-based vortex assisted matrix solid phase dispersion (VA-MSPD) approach

The modified IL-based VA-MSPD procedure used in this study is shown in Figure 2. A 1.5 ± 0.2 mg mass of pretreated plant tissue was transferred into an Agate mortar and 15 µL of the IL or MIL was added to the sample using a 25 µL gas tight syringe and dispersed using a pestle until all fragments of plant tissue were ground to fine particles. DMSO was added to the mixture in 15 µL aliquots and homogenized. The plant-IL-DMSO mixture was transferred into a qPCR tube and 15 µL of water was added to the mixture. The mixture was vortexed for 30 s followed by a centrifugation step for 30 s at 13000 *g*. The optimized volume ratio of IL: DMSO: water was 1:2:1 (v/v/v) and 1:4 (v/v) for MIL: DMSO. No water was added to the MIL: DMSO mixture. A 0.5 µL volume aliquot of the supernatant was placed into a qPCR tube for downstream analysis.

3. Results and discussion

3.1 IL-based direct solid-liquid extraction

ILs and MILs have been shown to efficiently lyse and extract DNA from complex biological matrices such as blood, bacterial cells, and plants [14,16,26]. The compatibility of hydrophobic ILs and MILs with qPCR makes downstream analysis efficient because DNA within the IL/MIL can be desorbed using the elevated temperatures of the qPCR thermocycling protocol [45]. In this study, one IL and two MILs featuring the trihexyl(tetradecyl)phosphonium cation ($[P_{6,6,6,14}^{+}]$) and multiple anions such as $[NTf_2^{-}]$, $[Ni(hfacac)_3^{-}]$ and $[Co(hfacac)_3^{-}]$ were chosen. Selection of the solvents is based on previous studies where they were used to extract DNA from plants and shown to be compatible with qPCR [25,27,45]. Extraction of DNA directly from plant tissue was based on applying the IL/MIL to a 1.0 mg cut fragment of dried tissue followed by incubation and recovery of the DNA-enriched solvent for qPCR analysis. Amplification was achieved for the $[P_{6,6,6,14}^{+}][NTf_2^{-}]$ IL, $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$ MIL and $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$ MIL (as shown in Figure 3) indicating that all solvents are capable of lysing and extracting DNA from very small portions of plant tissue.

The effect of incubation time on extraction efficiency was examined by carrying out extractions from 5 minutes to 24 h at 25 °C. Increasing the incubation time from 5 minutes to 24 h did not result in a significant change of the Cq values, as shown in Figure S1, indicating that there is no dependency of time on the extraction. However, to provide sufficient time, an incubation time of 1 h was chosen for subsequent experiments. The application of heat is common in many conventional plant cell lysis methods to facilitate efficient lysis of the plant cells within a short period of time. Therefore, the effect of temperature was examined in the IL-based direct solid-liquid extraction method. As shown in Figure S2, qPCR data did not reveal significant changes in the Cq values under varying temperature conditions indicating that it does not affect the amount of DNA extracted from small portions of plant tissue. It is possible that the amount of DNA present in 1 mg portions of dried plant tissue is sufficiently small such that an increase in incubation temperature and incubation time does not result in significant increases in the amount of DNA extracted. The volume of the extraction phase was also evaluated. A 10 μL volume of IL was used to extract DNA from 1 mg of plant to allow sufficient coating of plant tissue by the IL and this volume gave rise to best precision with the lowest standard deviation (as shown in Table S1). Extractions utilizing IL volumes lower than 10 μL (such as 8 μL and 9 μL) resulted in lower Cq values. However, these volumes were not sufficient to completely coat the plant tissue. Extractions utilizing larger volume of IL, such as 12 μL, resulted in higher Cq values which is likely due to dilution of DNA in the IL (Figure S3).

It is a common practice to dry plant tissue prior to DNA extraction to improve preservation of nucleic acids in the leaves for long term storage [5,46]. Therefore, the effect of different drying methods was investigated by keeping the weight of the plant tissue and the volume of the IL constant at 1 mg and 10 μL, respectively. As shown in Figure S4, tissue that was subjected to isopropanol treatment for 24 h resulted in higher amount of DNA extracted (lower Cq values). It was interesting to observe that DNA was extracted from the leaves when they were subjected to microwave treatment for 3 minutes. All tissues that were subjected to treatment prior to extraction gave rise to lower Cq values compared to the fresh tissue, indicating that more DNA is extracted from the treated tissue. Although the IL-based direct solid-liquid extraction method was compatible with fresh tissue, less DNA was detected (based on the higher Cq value) due to the high water content compared to the dry tissue. Fresh leaves were observed to lose more than 90 % of their weight due to drying. By taking the percentage of weight loss into consideration, the mass of fresh tissue that needs to be used is approximately 17 mg. A 10 μL volume of the IL was not sufficient to completely coat 17 mg of fresh plant tissue, resulting in inaccuracy when measuring the amount of DNA extracted. The volume of IL was kept to a minimum of 10 μL because higher volumes of IL have been shown to increase the Cq value as well as the standard deviation, as shown on Table S1.

The IL and MILs were successful in lysing plant cells and extracting DNA from small portions of plant tissue enabling successful qPCR amplification. However, direct addition of 0.5 µL of the IL into the qPCR buffer yielded an amplification efficiency of 87.8 %, which made reliable quantification of extracted DNA mass challenging. Furthermore, the standard deviation in the Cq values obtained when examining the effect of time, temperature, IL volume and sample pretreatment (Figures S1-S4) were high and exhibited poor repeatability. It was hypothesized that the IL viscosity may hinder the partitioning of high molecular weight DNA resulting in non-uniform distribution of DNA within the IL and higher standard deviations. Therefore, a new approach that incorporates two qPCR compatible co-solvents was explored in an effort to dissolve and dilute the solvents, achieve reduced viscosity, as well as mitigate any inhibitory effects caused in qPCR.

3.2 IL-based direct solid-liquid extraction employing a co-solvent

To reduce the viscosity of IL and MIL, DMSO and DMF were chosen as co-solvents as they are well-known qPCR compatible solvents that are commonly used to enhance amplification [47,48]. For an improved procedure featuring the co-solvent, fresh and air-dried fragments of plant tissue were cut into 4 symmetric sections and DNA from each cut leaf fragment was extracted using 15 µL of the IL for 1 h at room temperature. This was followed by the addition of an equal volume of co-solvent to dissolve the IL such that the ratio of the IL: DMSO was 1:1 (v/v). qPCR experiments were carried out in triplicate for each extraction to examine precision of the method. As the cut leaf fragments for the fresh and air-dried tissue represent the same sample, the average Cq for all qPCR experiments and each type of tissue was calculated. As shown in Table 2, the average Cq values for extractions with the fresh tissue was 26.92 ± 2.39 and that for dry tissue was 21.57 ± 0.84. Since dry tissue gave rise to less variability, subsequent studies were carried out using only air-dried tissue.

For optimization studies, different ratios of IL to DMSO were evaluated. The standard deviation of the Cq values obtained for the extractions was very high in case of the 1:2 (v/v) (as shown in Table S2) and 1:3 (v/v) ratios (data not shown) when compared to that of the 1:1 (v/v) composition. Similarly, 1:1 (v/v) and 1:2 (v/v) ratios of IL:DMF were tested, and the standard deviations were compared. As shown in Table S2, it was observed that the standard deviation associated with the Cq values when using 1:1 (v/v) and 1:2 (v/v) of IL-DMF mixtures resulted in higher standard deviation values. Although higher volumes of the co-solvent were used with the purpose of decreasing the IL viscosity, high standard deviations among the Cq values were still observed when the qPCR experiments were performed in triplicate, possibly due to extraction of other plant components which may affect amplification. Therefore, to eliminate any interfering components (e.g., chlorophyll) from the plant matrix, fresh leaves were immersed in ethanol for 12 h in an incubator at 37 °C (as shown in Figure S5) followed by DNA extraction from the pre-treated tissue.

3.2.1 Evaluating the effect of co-solvent and plant matrix on qPCR

To investigate the effect of co-solvent on qPCR, 10.2 pg of *A. thaliana* genomic DNA was spiked into 15 µL of the IL and incubated at room temperature for 1 hour. After incubation, the DNA-enriched IL was vortexed for 30 s with an equal volume of co-solvent such that the ratio of IL: co-solvent was 1:1 (v/v), followed by centrifugation for 30 s at 13000 *g.* A control experiment was carried out in which the same mass of plant genomic DNA was spiked into 15 µL of water and incubated at room temperature for 1 h. After incubation, an equal volume of water was added, vortexed for 30 s, and then centrifuged for 30 s. To investigate the effect of plant matrix on qPCR, 1.5 mg of plant tissue treated with ethanol was subjected to DNA extraction at room temperature for 1 h using 15 µL of the IL. After extraction, the DNA-enriched IL was vortexed for 30 s with an equal volume of co-solvent and centrifuged for 30 s at 13000 *g.* The final 1:1 (v/v) DNA enriched IL co-solvent mixture was then analyzed by qPCR. As observed in Figure 4, the Cq values obtained for plant genomic DNA in water and in 1:1 (v/v) IL-DMSO mixture were 24.34 ± 0.16 and 25.10 ± 0.53, respectively. A significant difference in Cq values was not observed for plant genomic DNA in water and the 1:1 (v/v) IL-DMSO mixture confirming that the 1:1 (v/v) IL-DMSO mixture did not inhibit the reaction. However, the Cq value was shifted by more than 5 cycles to 29.90 ± 0.63 in the 1:1 (v/v) IL-DMF mixture, as shown in Figure 4, compared to that of the control, indicating inhibition of the enzymatic reaction due to the presence of DMF. Similarly, the Cq value for the extracted plant DNA in the 1:1 (v/v) IL-DMF mixture was shifted by approximately 5 cycles compared to that in 1:1 (v/v) IL-DMSO mixture (as shown in Figure 4), further confirming that DMF inhibits the amplification reaction. Therefore, DMSO was chosen as the co-solvent for IL dissolution and dilution. It was also observed that the standard deviation of the Cq values was higher for extractions involving the plant tissue compared to the control experiments where DNA was spiked into the sample. This difference may be due to the variability arising from not grinding the sample with the IL as well as the static solid-liquid extraction approach resulting in non-uniform distribution of DNA within the IL. To overcome these challenges, a method involving mechanical grinding of the sample with the IL to facilitate simultaneous sample disruption and blending of the plant matrix with the IL in a homogenous fashion was developed.

3.3 Modified IL-based VA-MSPD approach

MSPD is an analytical procedure based on mechanical blending of the sample with a dispersant material in a mortar and pestle to maximize sample disruption and interaction [30,32,43]. Recent advances in MSPD-based applications have employed ILs for the extraction of synthetic dyes in condiments as well as phenolic acids and flavonoids in raw propolis [41,42]. However, studies that employ ILs or MILs in MSPD for the extraction of nucleic acids have not yet been reported. The modified IL-based VA-MSPD method employed in the study was based on grinding the homogenized plant material with the IL or MIL instead of using solid dispersive materials or co-sorbents. To develop an optimal IL-based MSPD method for extracting DNA from plant tissue, various parameters including tissue type, mass of plant tissue, type and volume of extraction solvent, volume of diluent as well as different plant dehydration methods were all assessed.

3.3.1 Sample pretreatment

Initial experiments employing IL-based VA-MSPD were conducted by grinding 1.5 mg of air-dried plant tissue with 25 µL of the IL, followed by recovering the mixture into a qPCR tube with 25 µL of DMSO. Due to its high viscosity, weighing or pipetting the IL was found to be challenging. Therefore, a 25 µL gas tight syringe was used to add the IL directly to the plant tissue that had been previously placed in the mortar. After grinding the sample with IL, DMSO was added in 15 µL and 10 µL respective aliquots and dispersed slowly with the pestle. The mortar was then tilted to facilitate separation of the solution from the plant tissue followed by recovery in a qPCR tube. As shown in Figure S6, the IL extract contained a green layer which was found to be repeatedly interfering with the recovery of the clear supernatant for qPCR. It was confirmed that the green layer was chlorophyll, as it glowed red under blue light at 470 nm [49]. Therefore, chlorophyll removal was deemed necessary suggesting the need for a sample pretreatment step.

Chlorophyll is a water insoluble pigment that can be easily removed with the use of organic solvents [50]. Hexane, absolute ethanol, methanol, and isopropanol were used for the removal of chlorophyll from fresh leaves by immersing them in the respective organic solvents at 37 °C in an incubator for 33 h. Residual solvent in the leaves was removed by placing them in a food dehydrator at 35 °C for 1 h and then recording the weight loss. All solvents, except for hexane, were observed to completely soak the leaves resulting in chlorophyll being leached from the tissues leaving an off-white color. As shown in Figure S7, ethanol treatment resulted in the highest weight loss and lowest RSD values. Previous studies have demonstrated the utilization of ethanol as a low-cost alternative to commonly used expensive methods for tissue preservation, such as lyophilization and liquid nitrogen treatment [46]. Ethanol preservation not only inactivates many nucleases and removes secondary metabolites but also makes the leaves more amenable for grinding and disruption [5,51]. Due to its ability in preserving the tissue and removing chlorophyll and secondary metabolites, ethanol was chosen as the optimal solvent for sample pretreatment.

The time taken for chlorophyll to leach into ethanol was observed to vary from leaf-to-leaf depending on the chlorophyll content. Therefore, to make the pretreatment process constant for every leaf fragment, fresh leaves were immersed in absolute ethanol for 12 h at 37 °C in an incubator to provide sufficient time for the chlorophyll to leach from the leaves (as shown in Figure S5). Any residual solvent in the leaves was removed in the food dehydrator at 35 °C for 3 h. Fresh leaves were observed to lose more than 90 % of weight when immersed in ethanol for 12 h compared to approximately 2 % weight loss when the dehydrated leaves were placed in the food dehydrator for 3 hours (Figure S8). After ethanol treatment, chlorophyll from the leaves was removed making the solution glow red under blue light (Figure S9a). The ethanol dehydrated leaves did not glow red under blue light illumination compared to the fresh leaves (Figure S9b and S9c) confirming all chlorophyll was removed from the leaves. Therefore, ethanol dehydrated leaves were used for subsequent experiments.

3.3.2 Optimization of IL: DMSO ratio

In optimization of IL:DMSO ratio, the mass of ethanol dehydrated tissue was kept constant at 1.5 mg and 25 µL of the IL was added directly to the plant tissue placed in an Agate mortar. The plant tissue was dispersed with the IL until it was ground to a fine powder. DMSO was added in two aliquots of 15 µL and 10 µL such that the final ratio of IL: DMSO was 1:1 (v/v). It was observed that an IL volume of 25 µL provided a higher volume for 1.5 mg of plant tissue and the viscosity of 1:1 (v/v) IL: DMSO mixture was not greatly reduced compared to that of the neat IL. Data from qPCR indicated a higher standard deviation for the Cq values of 3.02 cycles (Figure S10) which may be due to larger volumes of IL compared to the mass of the plant tissue, resulting in a more viscous mixture. Therefore, the volume of IL used for the extraction was decreased to 15 µL and the volume of DMSO added to recover the plant-IL mixture was increased to 30 µL. DMSO was added in 15 µL aliquots, followed by recovery into a qPCR tube. Next, 15 µL of water was added to further reduce the viscosity of the final mixture. After a brief vortexing step of 30 s and a centrifugation step of 30 s at 13000 *g,* a 0.5 µL volume of the clear supernatant was analyzed by qPCR. The representative photographs of the developed MSPD procedure are shown in Figure 5. Although the neat IL was not soluble in water, the IL-DMSO mixture was found to be miscible in water. The viscosity of the final IL-DMSO-water mixture was greatly reduced compared to that of the neat IL. qPCR data revealed that the decrease in IL viscosity resulted in a remarkable decrease in the standard deviation values from 3.02 to 0.24 cycles, as shown in Figure S11.

The DNA enriched IL-DMSO-Water mixture was stored at room temperature for 48 hours with the plant matrix to test if the addition of DMSO facilitates the extraction of additional DNA from the plant tissue. The Cq values were found to be constant for DNA in IL-DMSO-Water mixture even after 48 hours at room temperature (Figure S12), indicating that DNA extracted by the IL was stable and DMSO did not contribute to additional extraction from the plant tissue.

MILs have been previously used in MSPD for the extraction of pesticides from vegetables [31]. However, MSPD has never been combined with MILs for the extraction of DNA. As with the $[P_{6,6,6,14}^{+}][NTf_2^{-}]$ IL, the viscosity of the $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$ MIL and $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$ MILs also affected the qPCR data. By keeping the MIL volume constant at 15 µL, the volume of DMSO added was varied until the viscosity of the final mixture was greatly reduced resulting in a homogenous mixture. By varying the composition from 1:3 (v/v) MIL: DMSO to 1:3:1 (v/v/v) MIL: DMSO: water, the standard deviation for the qPCR experiments was reduced from 1.9 to 1.2 cycles (Figure S13). However, addition of water resulted in precipitation of the MIL. By increasing the ratio of MIL:DMSO to 1:4, the standard deviation was reduced to 0.34 (Figure S13) indicating that DMSO greatly reduces the viscosity of the MIL and improves repeatability. The optimum ratio of MIL:DMSO was found to be 1:4 for both the Ni and Co MILs.

When performing MSPD with the Co MIL, it was observed that the MIL blends well with the plant matrix due to its hydrophobicity. However, upon addition of DMSO, a precipitate was formed, as shown on Figure S14. The neat Co MIL was not observed to precipitate upon mixing with DMSO indicating that some components of the plant matrix may be responsible for precipitation when DMSO is added to the Co MIL. Due to this, the solution that was recovered consisted of mostly DMSO resulting in significant amounts of MIL being trapped in the plant matrix. Therefore, the Co MIL was not used for further experiments.

To quantify the mass of DNA extracted by the IL and Ni MIL under optimum conditions, standard curves were constructed by incorporating IL-DMSO-water and MIL-DMSO mixtures into the qPCR buffer. In qPCR, amplification efficiency is calculated by the slope of the standard curve and an amplification efficiency of 100 % relates to the ability of the DNA polymerase enzyme to double the amount of DNA in the reaction mixture with each cycle [52,53]. However, the amplification efficiency in practice is generally in the range of 90-105 % [53]. Amplification efficiencies lower than 90% or higher than 105% indicate the presence of inhibitors that ultimately affect quantification [45]. Therefore, it is important to investigate the influence that IL-DMSO-water and MIL-DMSO mixtures within the qPCR buffer have on amplification efficiency. As observed in Figure S15, the amplification efficiency associated with IL-DMSO-water and MIL-DMSO in the PCR mixture was found to be within 90-105%, representing a significant advantage of directly incorporating DNA-enriched IL-DMSO-water and MIL-DMSO mixtures into the qPCR master mix.

After optimizing the dehydration method, extraction conditions and composition of qPCR buffer, triplicate extractions were carried out using 1.5 mg of treated plant tissue and 15 µL of the IL and the Ni MIL. The optimum extraction conditions are summarized on Table 3. The $[P_{66614}^{+}][NTf_2^{-}]$ IL and $[P_{66614}^{+}][Ni(hfacac)_3^{-}]$ MIL extracted 2.87±0.28 ng of DNA/mg of plant tissue and 1.97±0.59 ng of DNA/mg of plant tissue, respectively. The performance of the IL-based

VA-MSPD method was compared with the NucleoSpin Plant II commercial kit, as shown in Table S3. The NucleoSpin Plant II commercial kit was found to extract DNA per milligram of plant tissue indicating that the mass of DNA isolated by the developed MSPD method is not as high, but can be considered significant based on the sample size and the amount of chemicals used. The mass of dried plant tissue used with the commercial kit was 20 mg compared to 1.5 mg that was used in the IL-based VA-MSPD method. Having a method that requires minute amounts of sample to extract sufficient amounts of DNA for subsequent downstream applications would be very useful, especially when analyzing ancient plant specimens. Additionally, the IL-based direct solid-liquid extraction method and IL-based VA-MSPD methods stand out for their miniaturized process, simplicity and low time requirement for the extraction compared to conventional methods that require an incubation period, large amounts of sample and solvents, and multiple centrifugation steps. Due to these advantages, the developed methods can be used as an alternative to kits. DNA extracted by both methods is of sufficient quantity for downstream applications involving DNA amplification such as loop-mediated isothermal amplification (LAMP) or qPCR. However, the IL-based direct solid-liquid extraction method would be more suitable for applications such as LAMP, which provides qualitative information whereas modified IL-based VA-MSPD method would be more useful for applications requiring precise quantitative information. The stability of DNA in the IL-DMSO-water and Ni MIL-DMSO mixtures upon storage at room temperature for 21 days was also investigated, as shown in Figure 6(a) and 6(b). Successful qPCR amplification was achieved after preservation using the IL/MIL-based VA-MSPD procedure, indicating that measurable amounts of DNA remain even after 21 days. However, as shown in Figure 6(a), the DNA mass in IL-DMSO-water was constant up to 48 hours followed by a decrease after this time period. Variability in the DNA mass may be attributed to inefficient amplification arising from the polymerase chain reaction. MIL-DMSO mixtures demonstrated greater DNA stability up to 14 days compared to IL-DMSO-water mixtures (Figure 6(b)). This agrees with previous studies which showed that salmon testes DNA and plasmid DNA stored with DNase I were stable within a hydrophobic MIL for up to 72 h at room temperature [54]. However, this study demonstrates that the longevity of the extracted plant genomic DNA can be extended by using MIL-DMSO mixtures, making this method ideal not only for extraction but also for storage prior to analysis.

4. Conclusions

This study is the first to integrate ILs and MILs into a modified VA-MSPD approach to enable cell lysis and extraction of genomic DNA from milligram fragments of treated *Arabidopsis thaliana* plant tissue. Compared to traditional methods that often incorporate tedious and laborious protocols, the present method enables DNA extraction with small amounts of sample and solvents while avoiding lengthy incubation steps to shorten the overall sample preparation time to a few minutes. DNA extracted by this approach was of sufficient quality and purity for subsequent nucleic acid amplification methods such as qPCR and could be preserved when stored at room temperature in IL- and MIL-DMSO mixtures. The hydrophobicity of the IL and MIL assisted in blending the extraction solvents with the dried plant matrix thereby facilitating cell lysis and subsequent DNA extraction, possibly through electrostatic interactions as well as hydrophobic interactions while also limiting their solubility in the qPCR buffer. An objective of the study was to understand more clearly the interactions that take place between ILs and MILs with the plant matrix and DNA. Future studies should seek to exploit the paramagnetic nature of MILs to facilitate their recovery and analysis in an entirely automated process. The versatility of the lysis/extraction approach and quality of recovered DNA makes it an appealing route for combination with downstream isothermal amplification methods that enable field analysis, particularly in plant disease diagnostics.

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Table 1. Chemical structures and formulas of ILs and MILs investigated in this study. The IL and two MILs are comprised of the [$P_{6,6,6,14}^+$] cation and three different anions.

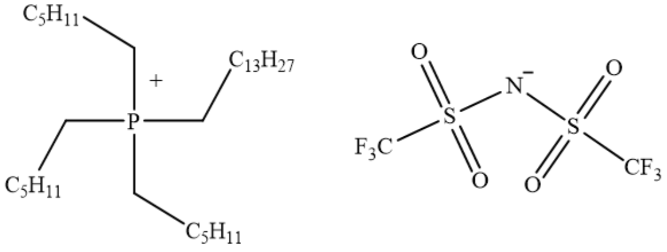
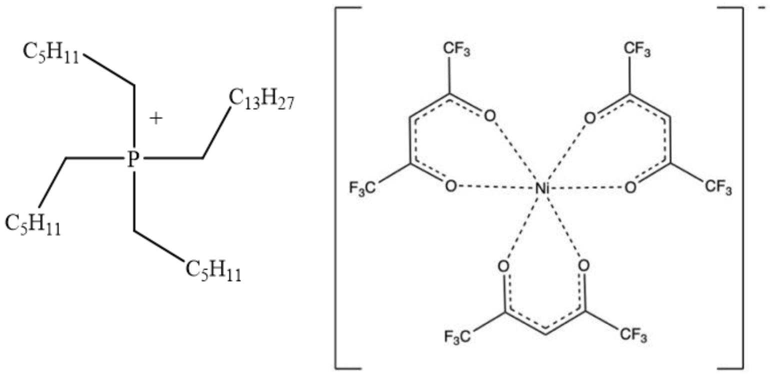
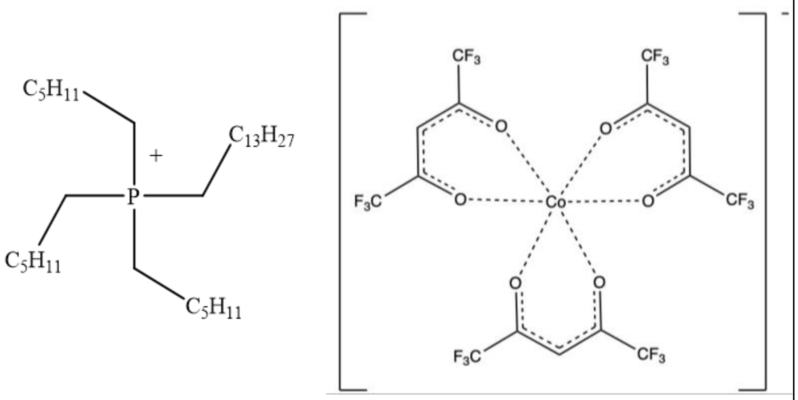
IL/ MIL	Chemical formula	Structure
1	[$P_{6,6,6,14}^+$][NTf_2^-]	
2	[$P_{6,6,6,14}^+$][$Ni(hfacac)_3^-$]	
3	[$P_{6,6,6,14}^+$][$Co(hfacac)_3^-$]	

Table 2. Influence of 1:1 (v/v) DMSO:IL mixture on the Cq values obtained when extracting DNA from *A. thaliana* fresh tissue and air-dried tissue using the $[P_{6,6,6,14}^{+}][NTf_2^{-}]$ IL.

Type of tissue	Air-dried tissue[a]				Fresh tissue[a]			
Cut leaf fragment	1	2	3	4	1	2	3	4
Mass of cut leaf fragment (mg)	1.2	1.5	1.3	1.4	6.8	5.7	6.6	5.1
Cq	21.76	22.14	20.77	21.60	25.64	25.71	29.77	26.53
SD	1.10	0.40	0.30	1.00	0.90	0.40	3.50	1.10

a. triplicate qPCR experiments were carried out for each extraction

Table 3. Optimum extraction conditions for the [$P_{6,6,6,14}^{+}$] [NTf_2^{-}] IL and [$P_{6,6,6,14}^{+}$] [Ni(hfacac)$_3^{-}$] MIL based VA-MSPD procedure using 1.5 mg of *A. thaliana* plant tissue treated with absolute ethanol for 12 h at 37 °C in an incubator shaker followed by the removal of residual solvent in the food dehydrator at 35 °C for 3 h.

IL/MIL used	Volume of IL/MIL (μL)	Volume of DMSO (μL)	Volume of water (μL)	Ratio of IL: DMSO: Water (v/v/v)
[P_{66614}^{+}] [NTf_2^{-}]	15	30	15	1:2:1
[P_{66614}^{+}] [Ni(hfacac)$_3^{-}$]	15	60	None	1:4

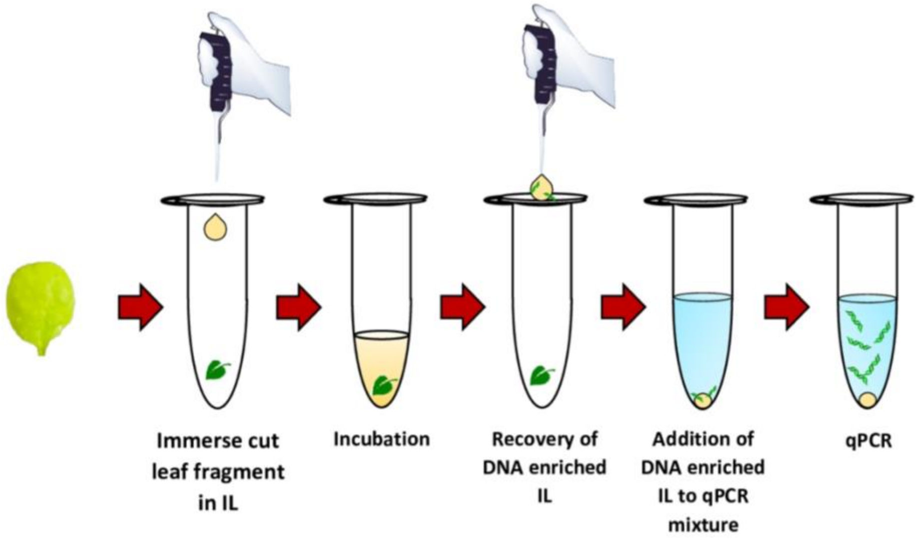


Figure 1. Schematic diagram for IL-based direct solid-liquid extraction of genomic DNA from 1 mg of air-dried *A. thaliana* plant tissue using 10 µL of $[P_{6,6,6,14}^+][NTf_2^-]$ IL or the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ or $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MILs.

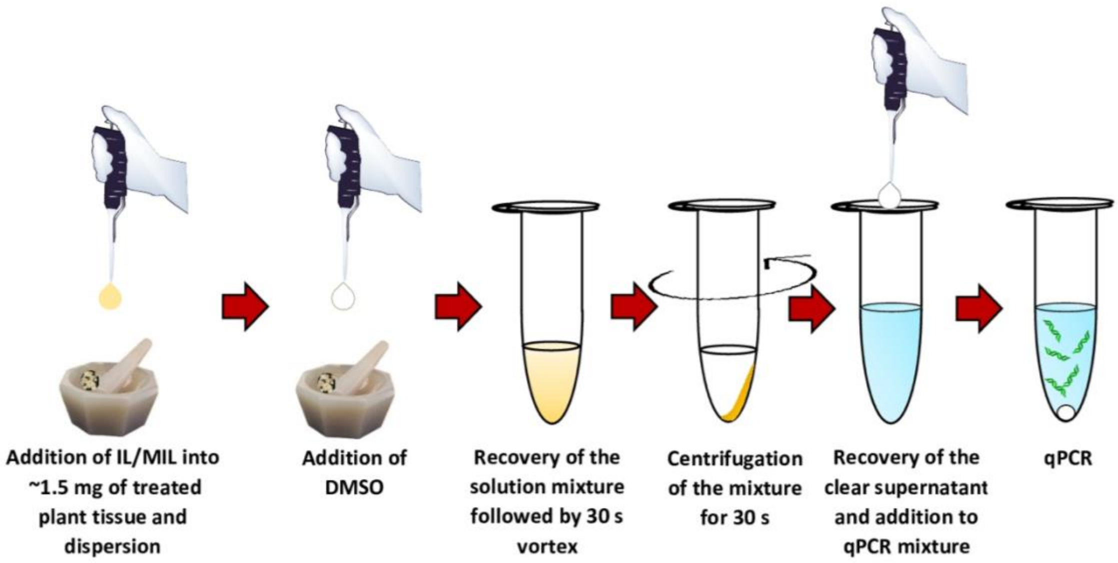


Figure 2. Schematic diagram of IL-based VA-MSPD for the isolation of genomic DNA from *A. thaliana* pretreated plant tissue using 15 µL of IL/MIL.

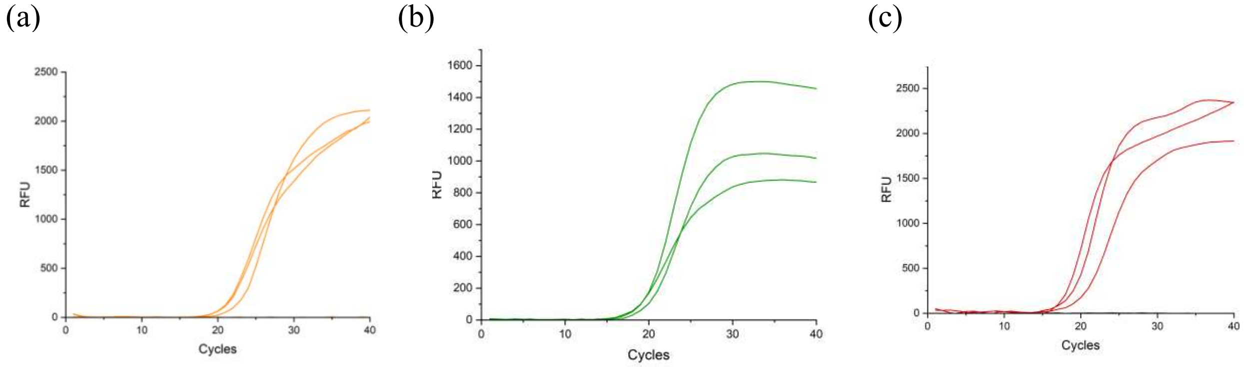


Figure 3. Amplification curves obtained by qPCR of the ITS target sequence of *A. thaliana* genomic DNA extracted by placing 10 μL of (a) [$P_{6,6,6,14}^+$][NTf_2^-] IL and (b) [$P_{6,6,6,14}^+$][$Ni(hfacac)_3^-$] MIL and (c) [$P_{6,6,6,14}^+$][$Co(hfacac)_3^-$] MIL on 1.0 mg of dried *A. thaliana* plant tissue for 1 h at 25 °C. All experiments were conducted in triplicate.

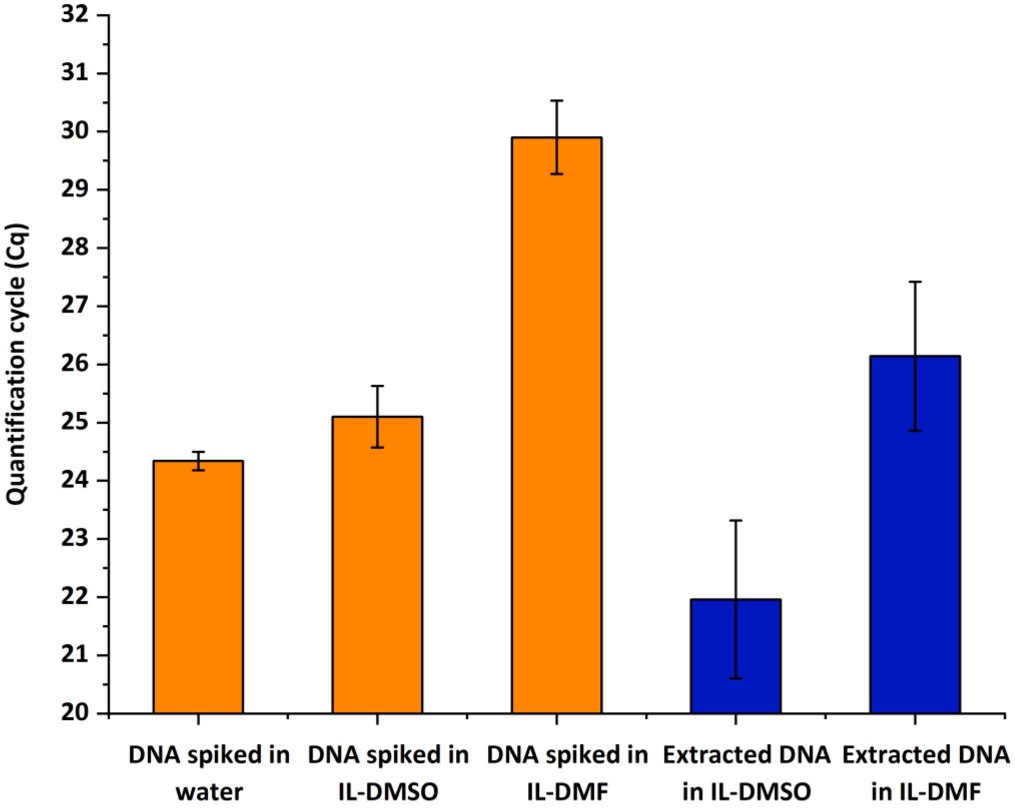


Figure 4. Influence of IL co-solvent mixtures and plant matrix on the amplification of ITS target sequence of *A. thaliana* genomic DNA. Control experiments (orange bars) were carried out by spiking pure *A. thaliana* genomic DNA into 15 µL of [$P_{6,6,6,14}^{+}$][NTf_2^{-}] IL and water and incubating at room temperature for 1 h followed by the addition of 15 µL of the co-solvent. Extraction experiments (blue bars) were carried out using 1.5 mg of *A. thaliana* pretreated plant tissue and 15 µL of [$P_{6,6,6,14}^{+}$][NTf_2^{-}] IL at room temperature for 1 h followed by the addition of 15 µL of the co-solvent. All experiments were conducted in triplicate.

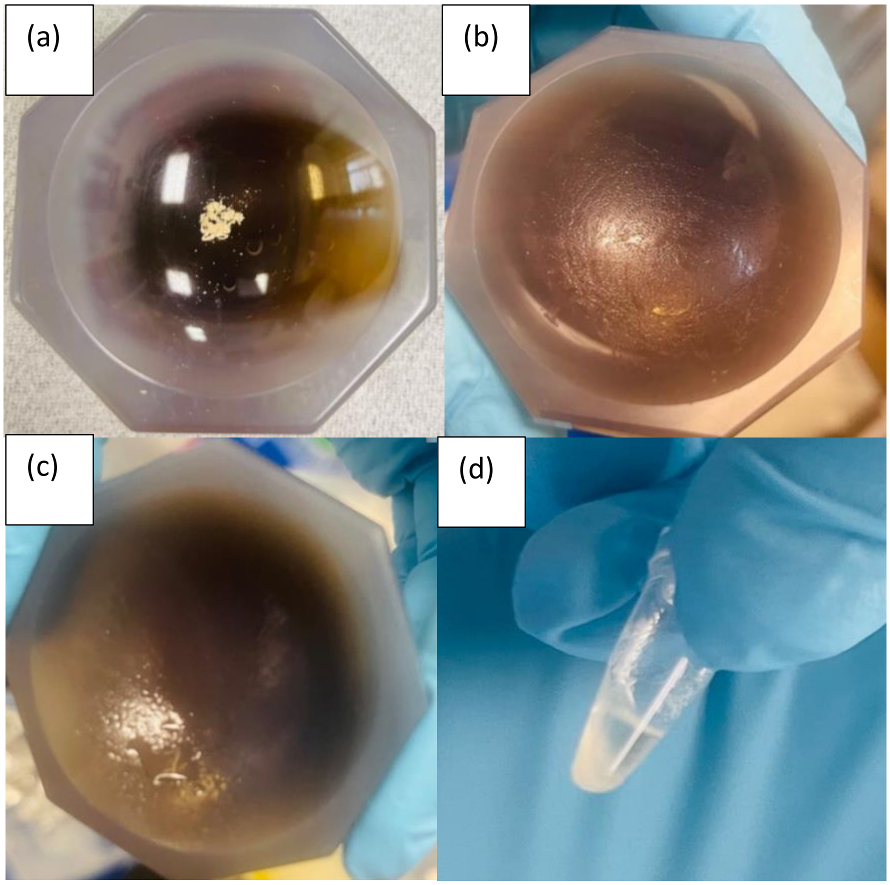


Figure 5. Representative photographs of various steps in the developed IL-based VA-MSPD procedure: (a) ground plant tissue in Agate mortar with the IL; (b) plant tissue dispersed with IL; (c) addition of DMSO for the recovery of plant-IL mixture and (d) clear supernatant that forms after centrifugation.

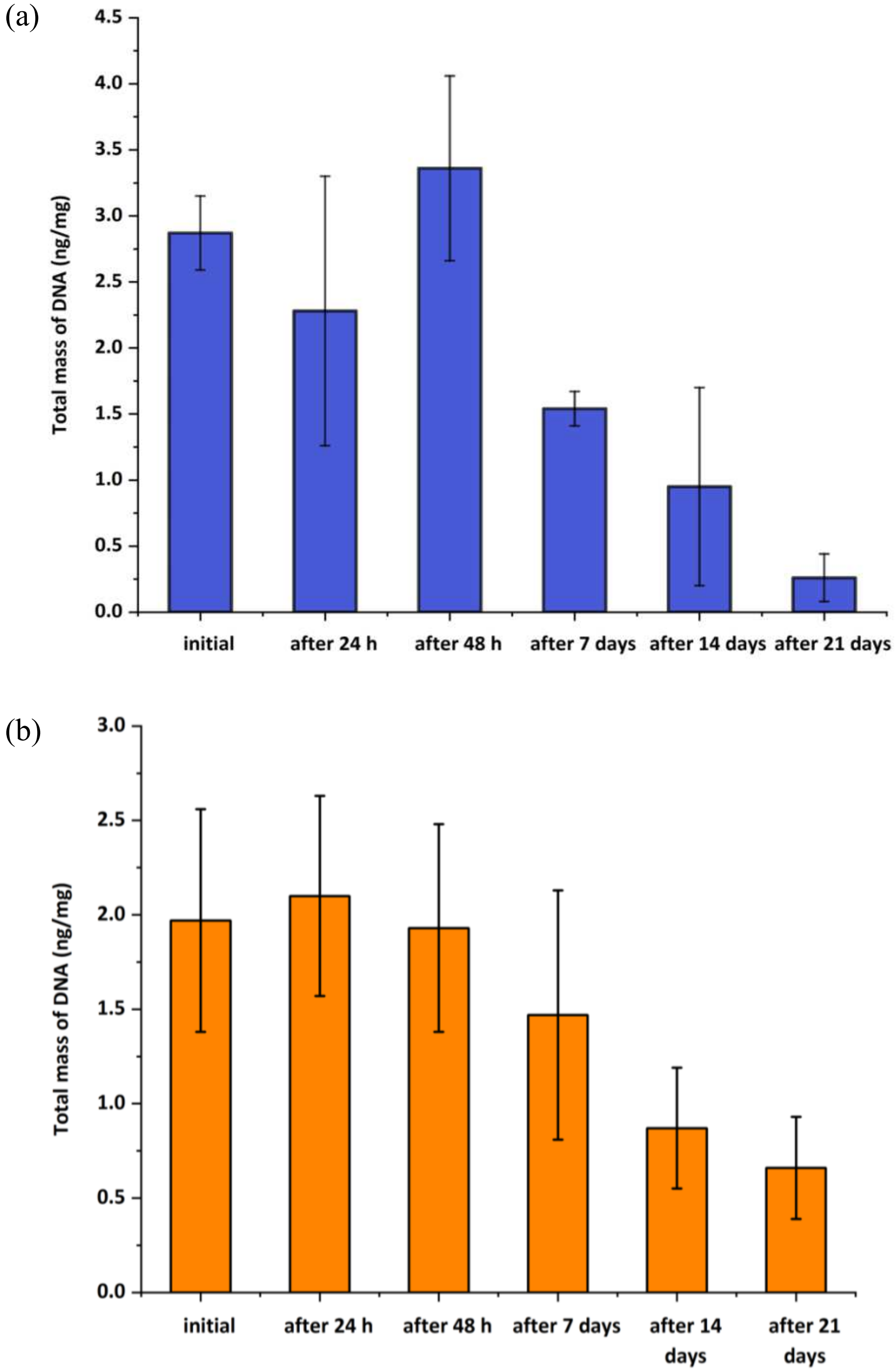


Figure 6. Stability of extracted DNA over time from 1.5 mg of *A. thaliana* treated plant tissue using 15 μL of (a) $[P_{6,6,6,14}^{+}][NTf_2^{-}]$ IL and (b) $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$ MIL. The MSPD procedure was used in the extraction and DNA was stored in IL-DMSO-water mixture and Ni MIL-DMSO mixture at room temperature. All experiments were conducted in triplicate.