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A universal protocol for the combined isolation of metabolites, DNA, long RNAs, small RNAs, and proteins from plants and microorganisms

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ABSTRACT:

Here, we describe a method for the combined metabolomic, proteomic, transcriptomic and genomic analysis from one single sample as a major step for multilevel data integration strategies in systems biology. While extracting proteins and DNA, this protocol allows also separation of metabolites into polar and lipid fractions, as well as RNA fractionation into long and small RNAs allowing for regulatory studies. The isolated biomolecules are suitable for analysis with different methods ranging from electrophoresis and blotting to state-of-the-art procedures based on mass spectrometry (accurate metabolite profiling, shotgun proteomics) or massive sequencing technologies (transcript analysis). The low amount of starting tissue, its cost-efficiency compared to the utilization of commercial kits, and its performance over a wide set of plant, microbial, and algal species such as *Chlamydomonas, Arabidopsis, Populus,* or *Pinus*, makes this method to a universal alternative for multiple molecular isolation from plant tissues.

INTRODUCTION:

Systems biological analyses aim for the integration of multilevel molecular data, such as metabolites, proteins, transcripts and genomic data and subsequent comprehensive multivariate statistical data analysis to reveal genome-wide associations and molecular phenotypes (Hood 2003, Weckwerth 2003). One of the major targets of systems biology is to use these statistical methods to establish data-driven models that are able to predict the non-linear behaviour of the system (Weckwerth 2011a, Weckwerth 2011b). Therefore, it becomes crucial to establish protocols which are not only concerned with post-extraction data integration but rather provide a mean to extract all molecular levels from one sample (Roume *et al.* 2012, Weckwerth *et al.* 2004), especially, when samples show high biological variation

and molecular fluctuation, or are limited in amount such as single cells, specific tissue, or fluids. Consequently, the different analyzed molecules (metabolites, DNA, RNA, and proteins) should be obtained from the same tissue to avoid bias and time effects.

Most of the integrative studies nowadays are based on an initial division of samples to perform different dedicated isolation methods. These workflows may induce biases and miscorrelations as different populations of proteins, RNAs, and metabolites may be found in the different tissue layers and organ parts (Eckert *et al.* 2012, Li *et al.* 2010, McConnell and Barton 1998). Moreover, sample limitation is often a problem for performing three-four targeted isolations for some experimental designs. In this work we describe a method for the combined sequential isolation of biomolecules (metabolites, DNA, RNAs – total, large, and small-, and proteins) without the need of employing commercial kits, which is a major step for multilevel data integration strategies in Systems Biology. This method has been tested and depurated in four different species and laboratories, ensuring its efficiency and reproducibility.

RESULTS:

Designing an efficient and cost-effective strategy for multiple extraction protocol

In our laboratory, we have developed analytical procedures for a high throughput description, quantitation, data integration, and statistical analysis of plant proteome and metabolome profiles and their systems biological integration to transcriptome and phenotype analyses. In these studies it is important to have a good overlap between the different omics datasets in order to reduce noise and gaps and increase the reliability of the analysis (Weckwerth 2011b). The basis for this integrated protocol was the procedure for the extraction of metabolites, proteins, and RNA from Arabidopsis tissues previously described by Weckwerth et al. (Weckwerth, Wenzel and Fiehn 2004). In brief, metabolites are extracted from grounded tissues with methanol:chloroform:water. After centrifugation, the supernatant is transferred to a tube with cholorform:water to perform phase separation, while pellets are saved for extracting proteins and nucleic acids by using hot SDS/phenol extraction method. This protocol is used for metabolite extraction in our labs on a daily basis. It works well in a broad range of species (from cyanobacteria to trees)(Furuhashi *et al.* 2011, Morgenthal *et al.* 2005, Morgenthal *et al.* 2007, Valledor *et al.* 2013, Wienkoop *et al.* 2008, Wienkoop *et al.* 2010) yielding polar and non-polar metabolites suitable for GC-MS or LC-MS. When starting from 50 mg of fresh weight, typical maximum intensities are greater than 1E8 in uHPLC-Orbitrap Mass Spectrometer when injecting 5 µL of the pellet resuspended in 150 µL of methanol. If a combined LC-MS and GC-MS approach is desired, we recommend to split the polar phase 80:20 (LC-MS:GC-MS) before drying. However, the yield of proteins and nucleic acids strongly depends on the plant species. Hot SDS/phenol solubilization works perfectly with herbaceous plants such as Arabidopsis. For rather recalcitrant tissues, such as Pine needles or certain Chlamydomonas strains, cleanness, yield, and integrity of pellets were below the requirements for optimal LC- MS based shotgun proteomics and NGS-based transcriptomics analyses.

To solve this problem, and considering that the metabolite isolation procedure is an established protocol, we focused on the subsequent pellet processing steps to increase the number and quality of isolated molecules. As the recovery and the purity of nucleic acids and

proteins in plant tissues strongly depends on blocking the oxidation events, mainly caused by polyphenols and other secondary metabolites, we modified the starting protocol by adding a pellet wash step with 0.75% (v/v) ß-mercaptoethanol in methanol immediately after pipetting out the metabolite fraction. This step increased the quality of the recovered proteins by hot SDS/phenol in recalcitrant samples, reflected by sharper bands after SDS-PAGE. However, protein degradation and low quality of RNA was observed. These facts, together with the drawbacks of working with hot phenol led us to consider an alternative approach.

As an alternative approach we modified the solubilization of the pellets formed after metabolite extraction, using a new buffer system based on a chaotropic salt (guanidine-HCL) and a combination of detergents (Tween 20/Triton X100) instead of SDS/phenol as previously described. The use of this new buffer reduced the degradation of proteins, and at the same time allowed the use of silica based columns for a differential fractionation of nucleic acids DNA and RNA (Figure 1).

Taking advantage of silica-based columns for nucleic acid extraction. Sequential fractionation of DNA, large RNA, and small RNAs.

The use of silica columns increased the recovery yield and the integrity of the nucleic acids when compared to previous protocols, showing similar performance to commercial RNA and DNA extraction kits (Tables 1 and 2). The described protocol was designed for coupling the basic fractionation principles of available protocols (Ausubel *et al.* 2002, Boom *et al.* 1990, Gjerde and Hoang 2009, Jiang *et al.* 2012), allowing for the fractionation of nucleic acids recovering high amounts of non-degraded biomolecules.

The presence of a chaotropic salt in the buffer at a very high concentration allowed the direct on-column precipitation of DNA while RNA is eluted with the flow through. To recover RNAs we decreased the dielectric constant of the solution by adding acetonitrile to the flow through, forcing the precipitation of RNAs on a new column. Columns were washed with an ethanol based solution, allowing the removal of the chaotropic salt and other contaminants before elution. If needed, on column RNase or DNase treatments can be applied to DNA or RNA samples respectively. Nucleic acids isolated this way showed no signs of degradation (Figure 2a,b), and quality suitable for demanding downstream applications such as next generation sequencing (Figure 2d).

The introduction of a size dependent RNA fractionation step was possible by the use of different proportions of acetonitrile (longer RNAs binds to the column in solutions with higher dielectric constant, Figure 2c). The use of acetonitrile in the RNA fractionation step was more effective and reproducible than using ethanol, acetone or other solvents recommended in commercial kits (i.e. Nucleospin Tri-Prep and miRNA (Macherey-Nagel), AllPrep (Qiagen), MirVana(Life-Technologies)). At the same time, the use of acetonitrile reduced on-column protein precipitation thereby increasing recovery.

Depending on the species this protocol yields up to 21 µg of high molecular weight DNA per 50 mg of fresh weight (Table 1). The obtained yields were slightly below those obtained by using a

dedicated commercial isolation kit (Table 2). The use of silica columns allows the purification of a DNA free of contaminating RNA and proteins with adequate A_{260}/A_{280} ratios which should be ~1.8. The observed A_{260}/A_{280} ratios were better than those obtained with commercial kits. The isolated DNA following this method is suitable for digestion, PCR-based amplification, and other analytical procedures such as the quantitation of nucleobases in DNA (Hasbún *et al.* 2008) for determining DNA methylation. For cases like quantitation of DNA methylation, in which the presence of RNA traces is critical, we recommend to perform an extra RNase treatment.

Two different workflows were tested for isolating RNA. The quick total RNA procedure routinely yielded more than 10 µg of RNA (Table 1) when starting from 50 mg FW of tissue. The values obtained by this protocol are, in general, smaller than those obtained from commercial kits, except for the recalcitrant species *Pinus radiata* (Table 2). For samples of low or moderate weight (<50 mg FW), which will not saturate the DNA column, there is no need of an extra DNase treatment in this protocol (No traces of DNA were detected in Agilent Bioanalyzer, Figure. 2d). However, testing the presence of DNA traces when working with new species is recommended. If needed, an on-column DNase treatment can be performed. The isolated RNAs were suitable for any application in molecular biology ranging from hybridization to next generation sequencing, as demonstrated by RNA integrity analyses (Figure 2b, d). The RNA fractionation procedure, employing different concentrations of acetonitrile (Figure 1c), provides a cost effective way to separate long and small RNAs with a high grade of purity (A_{260}/A_{280} ratios~2.0; Table 1) and integrity (gel electrophoresis and Agilent Bioanalyzer; Figure 2c, d).

Protein purification

Proteins were purified from the flow through fraction employing a classic method based on phenol purification (Valledor and Weckwerth 2013). This method yielded 0.5-1.8 mg of protein from 50mg FW, ranging from 10 µg protein/mgFW in pine needles to 38 µg protein/mgFW in Arabidopsis leaves, and is presented in a denatured form precipitated in a pellet. These amounts are enough for high throughput proteomic analyses based on 2-DE PAGE or LC-MS/MS analyses (Figure 3c, d), with more than 400 spots resolved on 7 cm gels and 750-950 proteins detected in a 2 h gradient Orbitrap-MS run (LC-MS set up and runs were performed according to Valledor and Weckwerth (2013). The purifications of proteins following RNA extraction after using a commercial kit yielded 0.27-0.46 mg of proteins, being significantly lower than our method. Proteins isolated from commercial kits were also suitable for proteomic analyses, as it is widely described (Roume *et al.* 2012).

The usage of methanol:chloroform for metabolite extraction provided sharp chromatograms (Figure 3c,d). This solution facilitates membrane disaggregation due to the solubilization of lipids, increasing the recovery of membrane proteins. Chloroform also reduces the degradation due to the quick denaturation of all protein species, including proteases altogether providing a good balance of cell protein representation. The use of acetonitrile instead of acetone (used in some commercial kits) for reducing the dielectric constant increased the protein recovery, however, when using high concentrations of acetone we observed that some protein

precipitate retained in the silica columns. Purified proteins are suitable for downstream application ranging from SDS-PAGE and immunoblotting to two dimensional gel electrophoresis, or high-throughput characterization and quantitation using a shot-gun proteomics approach based, i.e., on nano-uHPLC-Orbitrap/MS analyses. All of these analyses can be directly matched.

DISCUSSION:

In this work, we describe an integrative approach for the sequential isolation of biomolecules (metabolites, DNA, RNAs, and proteins) with high quality and quantity for high-throughput omic analyses. Using this approach we were able to generate multi-omic datasets from small amounts of material, which will allow a better integration of data, reducing biases and thus increasing confidence, in systems biology studies.

The concept of this approach, was published by Weckwerth and coworkers in 2004 and can be considered a standard procedure for metabolite and protein isolation which has been applied in many studies, having enabled the integrative analysis of proteins and metabolites (Morgenthal *et al.* 2005, 2007; Wienkoop *et al.* 2008,2010; Valledor *et al.* 2013). Recently, Roume et al. (2012) extended this integrative protocol to mixed microbial samples, also allowing the study of proteins, and RNA. This approach relies on the initial metabolite separation following a procedure similar to Weckwerth et al. (2003), and a subsequent isolation of proteins and nucleic acids from pellets using commercial kits. The protocol presented here has major advantages over Roume et al. (2012): firstly, the yields are almost 4-fold higher for nucleic acids and 20-fold for proteins when comparing LAO-Enriched microbial communities to our Chlamydomonas results (Table 1) while also providing high quality nucleic acids suitable for deep sequencing; secondly, the protocol is more cost-effective since it is not based on expensive commercial kits; thirdly the protocol is faster.

The advantage of our method compared to other alternatives relies on two specific steps. On one hand the solubilization of the pellet in a buffer that contains a high concentration of a chaotropic salt and a combination of two nonionic surfactants allows the complete pellet rehydration and the direct binding of DNA to the first silica column. On the other hand we propose the use of acetonitrile, instead of acetone or any alcohol as recommended by commercial kits, for RNA binding to silica columns. The use of acetone for isolating RNAs in some samples causes a visible protein precipitation in the silica column reducing protein yields and RNA quality. On contrary, proteins are soluble in acetonitrile, resulting in improved protein recovery and cleanness of RNAs.

This method was easily implemented in four laboratories by researchers with and without experience on purification protocols. During the testing period a broad range of species (*Cyanothece, Chlamydomonas, Lemna, Arabidopsis, Oryza, Populus, Pinus, Eucalyptus)* and tissues (leaf, root, lyophyllized tissues) were assayed. The quality of the purified molecules, in all species, were in line with the results shown above, however yields strongly depended on plant species and organ.

The purity of recovered nucleic acids, based on A_{260}/A_{280} ratios, isolated with this protocol is better than those values obtained by commercial kits. This can be explained by the fact that

the initial extraction of metabolites strongly purifies the samples, removing most of the compound that may interfere with the commercial buffers. As expected, the employ of a specific DNA isolation kit (Qiagen DNeasy) resulted in better yields than obtained by this multiple protocol when eluting the columns once. When eluting two times, yields increased surpassing these values. Focusing on RNAs our method performed better than commercial kits for Pinus and Arabidopsis, being these values more than 50% higher, for the other tested species the yields could not improve the commercial kits, but the A_{260}/A_{280} ratios are generally better. It must be considered that TriPrep kit was not designed for plant tissues, being this reflected in smaller yields and higher degradation of large RNAs with the exception of Poplar and Chlamydomonas samples. On the other hand the amount of purified protein is significantly higher while using the described protocol. In the case of commercial kits, the low yields obtained in Arabidopsis and Poplar may be limiting for demanding methodologies such as 2D-PAGE in large (>17 cm) IPG strips and gels. Overall, the integrated protocol yielded values comparable to independent commercial kits or slightly smaller, but in all cases the amount of isolated biomolecules is not limiting for any high-throughput approach.

We can consider this method universal, since it works in a broad range of organisms without any modification of the described protocol, and it can be incorporated in any laboratory since it requires minimum equipment being considerably cheaper than other available alternatives. This protocol has been tested in four different laboratories, with different backgrounds and skills in molecular biology, always with satisfactory results in a broad range of plant species. This fact demonstrates that this protocol is easy to implement in any lab, and also robust with a wide application range. Furthermore, the design of this protocol together with the availability of 96-well silica columns will allow an easy downscaling of buffer and sample volumes for setting up workflows for high throughput biomolecule purification starting from the same sample. The routine capability of extracting metabolites, DNA, RNAs, and proteins from the same small amount of sample overcomes the present limitation in multi-omics studies that needs enough sample to perform individual isolation of biomolecules. The reduction of analytical biases derived from using the same initial tissue, together with the ability to cope with experimental designs based on minimum amount of materials provides a major advance for Green Systems Biology.

EXPERIMENTAL PROCEDURES:

Chemicals and reagents

The employed solvents were LC-MS grade and bought from different vendors. All chemicals were molecular biology or MS grade and bought from Sigma. All plastics were purchased from Eppendorf. Multi layers silica columns were bought from Epoch Life Science (Econospin, 1940-250).

Plant material

Expanded leaves were taken from healthy Poplar (*Populus trichocarpa*), Radiata pine (*Pinus radiata*), and Arabidopsis (*Arabidopsis thaliana*) plants were sampled. *Chlamydomonas reinhardtii* (CC503 strain) was cultured in TAP media until $1x10^6$ cells/mL and harvested by

centrifugation. All materials were immediately frozen in liquid nitrogen after sampling, and kept at -80°C until use. Each sample and replicate was constituted by 50 mg of fresh weight.

Metabolites extraction

Eight hundred microliters of cold (4°C) metabolite extraction buffer (methanol: chloroform:water 2.5:1:0.5) were added to 50 mg of tissue (frozen, fresh weight) and grinded in a bead mill homogenizator (Retsch MM400) for 30 s. Samples were centrifuged 20.000 x *g* for 6 min at 4°C and the supernatant were transferred to 2 mL microcentrifuge tubes containing 800 µL of phase separation mix (chloroform:water 1:1). Pellets, containing proteins and nucleic acids were immediately washed with 1 mL of 0.75% (v/v) ß-mercaptoethanol in 100% methanol. Tubes with metabolites were centrifuged at 10000 x *g* and 5 min at room temperature. Two phases should be clearly defined with a sharp interface. Polar and non-polar metabolites, upper and lower layers respectively were transferred to new tubes. These two fractions were washed again with 300 µL of phase separation buffer, centrifuged, and fractioned again. Polar and non-polar layers were saved to new tubes and dried on speed-vac. Tubes with pellets were centrifuged at 20.000 x *g* for 6 min at 4°C and the supernatant was discarded. Pellet washing steps were repeated once, and finally pellets were air dried.

Nucleic acids extraction

Pellets were dissolved in 400 µL of pellet solubilization buffer (PSB) (7M Guanidine HCl, 2% (v/v) TWEEN 20, 4% (v/v) TRITON-X100, 50 mM Tris, pH 7.5, 1% (v/v) ß-mercaptoethanol) and incubated at 37°C in a thermal shaker until complete solubilization (usually 20-30 min). Samples were pipetted to a new tube and centrifuged at 14000 x *g* for 3 min to remove insoluble particles. After centrifugation the supernatants were transferred to new silica columns to bind DNA. After 1 min of incubation columns were centrifuged at 10000 x g for 1 min and the flow through were transferred to a new column. Columns with bound DNA were reserved for washing steps until RNA isolation was finished. The flow through containing RNA and proteins was immediately mixed with 300 µL of acetonitrile for total RNA extraction (for RNA fragmentation see below). The mix was transferred to a new silica column, incubated for 1 min, and centrifuged at 10000 x g for 1 min. The flow through was transferred to a new 2 mL microcentrifuge kept at 4°C or -20°C until protein isolation. Columns with bound DNA or RNA were washed with 750 µL of WB1 (2 mM Tris pH 7.5, 20 mM NaCl, 0.1 mM EDTA, 90% ethanol), and then centrifuged at 12000 x g for 2 min. The flow through was discarded and columns were washed with 750 µL of WB2 (2 mM Tris pH 7.5, 20 mM NaCl, 70% ethanol) and then centrifuged 12000 x g for 2 min. Flow throw was discarded and columns were centrifuged again for 1 min at 14000 x g to completely dry the membrane. Nucleic acids were eluted from the column in 50 µL of DNA elution buffer (for DNA samples, 10 mM Tris, pH 8.0, 1 mM EDTA) or DNase- and RNase free water. For maximum yields columns can be eluted twice with 75 or 100 µL of water. Samples can be later concentrated on speedvac.

For independent isolation of long and small RNAs, the flow through coming from the DNA column was transferred to a new tube and its volume was adjusted to exactly 600 µL with PSB. Then 175 µL of acetonitrile were added and mixed thoroughly to adjust binding conditions. The mixture was then transferred to a new silica column to bind long RNA, incubated for 1 min, and then centrifuged 12000 x g for 2 min. The flow through containing small RNAs and

proteins was mixed with 125 µL of acetonitrile and transferred to a new silica column. Samples were incubated for 1 min, and then centrifuged 12000 x g for 2 min. The last flow through was transferred to a new 2 mL microcentrifuge tube and kept at 4°C or -20°C until protein isolation. Columns containing long and small RNAs were washed with WB1 and WB2 as previously described.

An on column digestion of RNA and DNA can be performed on column after the first washing. DNase I and RNase A powder should be reconstituted following the vendor instructions, 30 kunitz of DNase I or 5 units of RNase A were added to each column in a volume of 50 µL and incubated for 20 min at room temperature. After nuclease treatment the WB1 step was repeated before continuing the protocol with the WB2.

Protein extraction

Proteins were purified from the flow through by using a phenolic extraction. 550 µL of phenol (Tris-Buffered pH 8) and 600 µL of water were added to each tube containing the saved flow through. Samples were mixed in a vortex for 2 min at room temperature, and then centrifuged at 10000 x g at room temperature for 5 min. Supernatant, containing the phenolic phase, was transferred to a new 2 mL microcentrifuge tube containing 600 µL of PWB (0.7M Sucrose, 50 mM Tris-HCl pH 7.5, 50 mM EDTA, 0.5% ß-mercaptoethanol, 0.5% (v/v) Plant Protease Inhibitor Cocktail from Sigma) and mixed thoroughly by vortexing. After centrifugation at 10000 x g at room temperature for 5 minutes, upper phenolic phase was transferred to a new tube. Proteins were precipitated by adding 1.5 mL of 0.1 M ammonium acetate, 0.5% ß-mercaptoethanol in methanol and an overnight incubation at -20°C. Samples were then centrifuged at 10000 x g for 15 min at 4°C. Supernatant was removed and protein pellets were washed with acetone in an ultrasound bath until complete disaggregation of the pellet. Proteins were precipitated by centrifugation (10000 x g for 15 min at 4°C) and acetone was removed. Washing step was repeated three times. Protein pellets were allowed to air dry and resuspendend in adequate buffer for SDS-PAGE or LC-MS analyses.

Dedicated isolation of biomolecules using standard procedures

DNA and RNA were isolated by using Qiagen's Plant DNeasy and RNeasy (Düsseldorf, Germany). Small RNAs were purified with miRNA isolation kit from Macherey Nagel (Düren, Germany). Proteins were recovered from RNA extraction buffer by acetone precipitation. All procedures were conducted according to manufacturer's instructions.

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AUTHOR CONTRIBUTIONS

The extraction protocols were optimized as follows: metabolites (W.W.), Pellet solubilization and DNA isolation (L.V.), Long and Small RNAs fractionation (M.E.), total RNAs (M.M., M.E.), and protein purification and analysis (M.M, L.V.). M.J.C. provided Agilent bioanalyzer analyses and E.N. Orbitrap runs. L.V. designed the protocol and supervised its development and tuning. All authors participated in writing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

TABLE 1

Table 1: Mean yields achieved with this protocol corresponding to an initial amount of 50 mg of fresh weight in the tested plant species. Yield column indicates the average yield (n=3) in micrograms, while A_{260}/A_{280} range the minimum and maximum ratios. Columns were eluted once with 50 µL of deionized water. Values in brackets of DNA samples indicate the amount of DNA recovered when using 150 µL of water for column elution.

	DNA		Total RNA		Long RNA		Small RNA		Protein
	Yield	A_{260}/A_{280}	Yield	A_{260}/A_{280}	Yield	A_{260}/A_{280}	Yield	A_{260}/A_{280}	Yield
Poplar	7.8 (9.2)	1.4-1.6	12.6	1.7-2.2	19.6	1.7-1.9	4.3	0.8-1.0	1190
Pine	4.9 (5.7)	1.4-1.7	10.1	1.6-1.8	4.0	1.6-1.8	3.9	0.8-0.9	503
Arabidopsis	11.8 (21.0)	1.5-1.8	30.6	1.9-2.0	58.2	1.7-2.0	7.4	0.9-1.3	1903
C. reinhardtii	5.6 (6.4)	1.6-1.8	13.4	2.2-2.4	20.4	1.7-1.9	5.1	0.8-0.9	1821

Table 2: Mean yields achieved with this protocol corresponding to an initial amount of 50 mg of fresh weight in the tested plant species using different commercial kits. Yield column indicates the average yield (n=3) in micrograms, while A_{260}/A_{280} range the minimum and maximum ratios. Columns were eluted once with 50 µL of deionized water.

	DNA (Qiagen DNeasy)		Total RNA (Qiagen RNeasy)		Long RNA (MN Tri-prep)		Small RNA (MN Tri-prep)		Protein
	Yield	A_{260}/A_{280}	Yield	A_{260}/A_{280}	Yield	A_{260}/A_{280}	Yield	A_{260}/A_{280}	Yield
Poplar	1.5	1.4-1.6	14.7	1.8-1.9	15.3	1.1-1.4	4.8	1.2-1.4	389
Pine	6.1	1.1-1.3	2.6	1.1-1.2	0.7	1.1-1.7	1.8	1.1-1.2	461
Arabidopsis	12.1	1.5-1.8	12.2	1.7-1.8	17.3	1.1-1.72	2.8	1.1-1.2	269
C. reinhardtii	20.1	1.5-1.6	27.3	1.9-2.0	58.7	1.6-1.7	7.5	1.5-1.6	394

FIGURE LEGENDS

Figure 1: Protocol workflow. The different steps of this protocol are indicated in colors. Timings included in this figure have been calculated for batched of six samples, however it is possible to handle 12 or 18 samples per batch.

Figure 2: Nucleic acids analysis. (a) Electrophoresis of the DNA extracted from the different plant species on a 0.8% (w/v) agarose gel. In each lane 100 ng of DNA was loaded. (b) Non denaturing electrophoresis of the RNA extracted from the different plant species on a 1.2% (w/v) agarose gel. In each lane 150 ng of total RNA was loaded. (c) Effect of the acetonitrile concentration in the fractionation of long and small RNAs. The different tested volumes of acetonitrile are shown above the lanes. 200 ng of long RNA and 150 ng of small RNA isolated from pine samples were used. Fractionated RNAs were loaded on 0.8% (w/v) agarose gel. (d) Analysis of RNA integrity from random samples covering total, long, and small RNAs performed on an Agilent 2100 bioanalyzer. The amount of RNA is depicted in fluorescence units (FU) and the RNA Integrity Numbers (RIN) are indicated on the figure.

Figure 3: Isolated proteins and metabolites. (a) 2DE of *Pinus radiata* extract. 80 µg of protein was passively loaded onto 7 cm pH 5-8 IPG strips, and then proteins were resolved according to its MW employing a 13.5% SDS-PAGE gel. More than 350 spots were resolved after Sypro Rubi (Biorad, USA) staining. (b) Base peak representation of trypsin digested samples corresponding to Chlamydomonas, Arabidopsis, Poplar, and Pine (from top to bottom). A total of 624, 852, 631, and 467 proteins were respectively identified using SequestHT algorithm and genome-based protein databases specific for each species. Peptides were separated in an Ascentis Express Peptide ES-C18 15 cm HPLC column within 90 min linear gradient from 5 to 40% of 0.1% formic acid in 90% acetonitrile and measured with LTQ-Orbitrap XL (Thermo Electron). Typical chromatrograms of the (c) polar and (d) non polar metabolites of Chlamydomonas, resolved on a GC-MS instrument.

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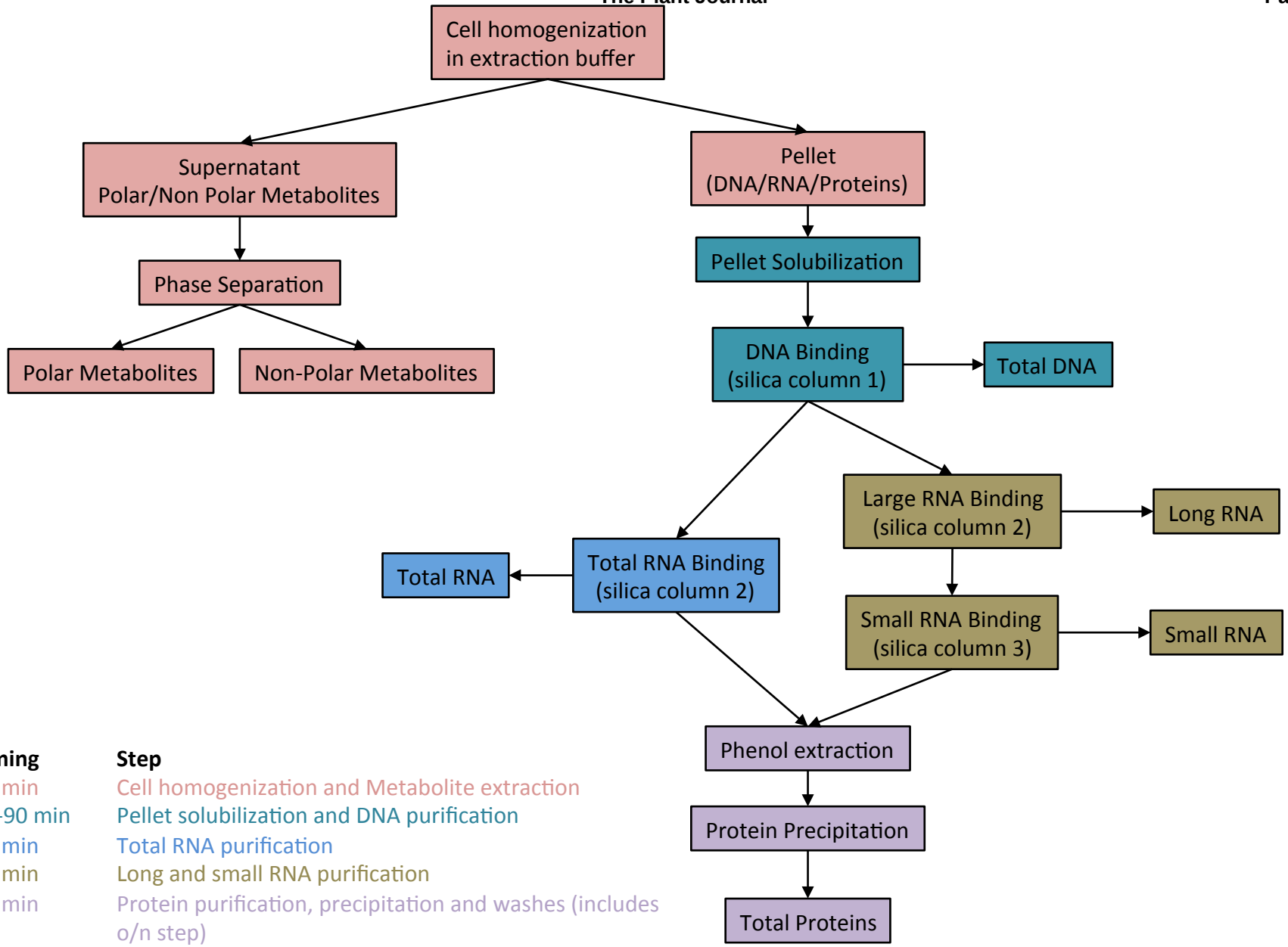
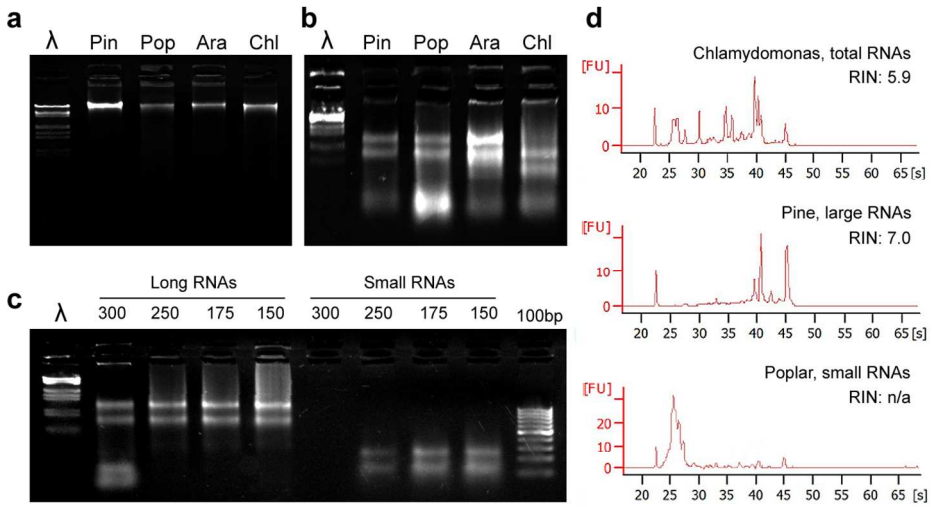


Timing	Step
90 min	Cell homogenization and Metabolite extraction
60-90 min	Pellet solubilization and DNA purification
30 min	Total RNA purification
80 min	Long and small RNA purification
80 min	Protein purification, precipitation and washes (includes o/n step)



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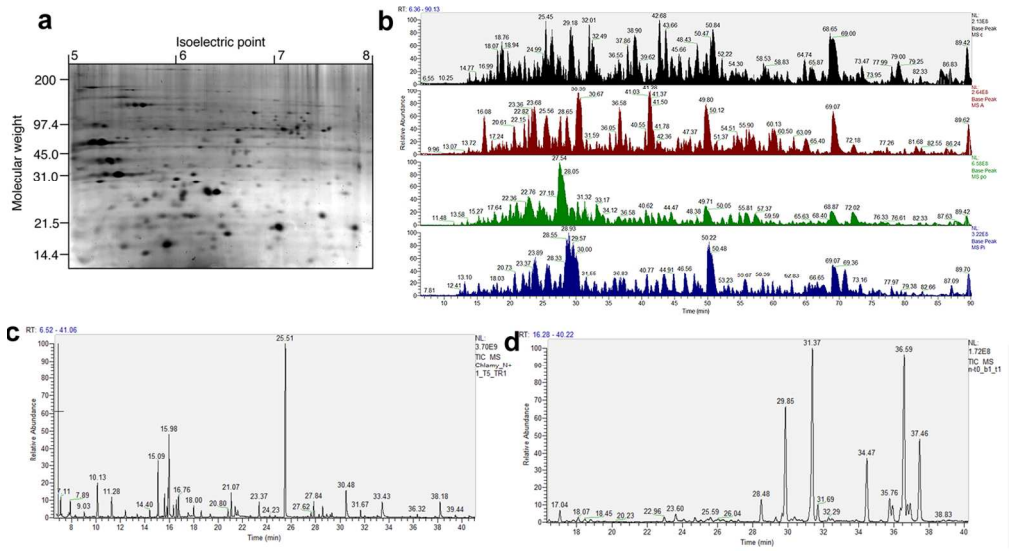

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