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Title: Optimizing 1D ¹H-NMR profiling of plant samples for high throughput analysis: extract preparation, standardization, automation and spectra processing

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Online resource 1. Plant culture and sample preparation protocol for the wheat spikelet NMR experiment

Plant culture conditions and sample harvest

Triticum durum (durum wheat) cv. Sculptur plants were cultivated in a greenhouse. Spikelet without awns were harvested at 5 or 14 days after flowering (DAF), frozen in liquid nitrogen and stored at -80°C before freeze-drying. To evaluate the impact of a pathogen on the stability of plant methanolic extracts, some wheat kernels were inoculated with *Fusarium*, a plant pathogenic fungus, at the flowering stage. A biological replicate was constituted of 5 spikelets at 5 DAF and 2 spikelets at 14 DAF.

Sample preparation and extraction

Sample weight: 40 ± 1 mg DW in 2 ml polypropylene microtube with conical bottom with skirt (e.g. 2.0 ml SC Micro Tube protein LB from Sarstedt).

Chemical products and extraction solutions:

- D₂O 99.9%D
- NaOD (99.5% D) 40% w/w
- DCl (99.8% D) 11.8 N
- 1 M NaOD in D₂O with 0.01% w/v NaN₃.
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- 58 mM TMSP (3-trimethylsilyl propionic-2,2,3,3-*d*4 acid, sodium salt, 98%D) in D₂O.
- MeOD-*d*4 99.8%D, (HOD + D₂O) < 0.03%
- 98 mM EDTA-*d*12 in D₂O at pH_{apparent} 8.7. Dissolution of acidic form of EDTA-*d*12 in D₂O with drops of NaOD 1 M solution to pH_{apparent} 8.7. Solution kept at 4°C for 1-2 months
- 90 mM phosphate (KD₂/K₂D PO₄ in D₂O) buffer solution at pH_{apparent} 6, containing 11 mM EDTA-*d*12

Extraction and pH adjustment

Manual Extraction: add 750 µl MeOD-*d*4 (with positive displacement pipette) under a hood (with gloves, glasses and protective clothes). Vortexing for 1 min at maximum speed. Then add 750 µl of buffer solution at pH_{apparent} 6 (phosphate buffer KD₂/K₂D PO₄ 90 mM, 11 mM EDTA-*d*12). Vortexing for 1 min at maximum speed.

Automated Extraction: add with a robot 1,500 µl of a solution at pH_{apparent} 6.0 (50/50, v/v MeOD-*d*4 / phosphate buffer KD₂/K₂D PO₄ 90 mM, 11 mM EDTA-*d*12) under a hood (with gloves, glasses and protective clothes). Vortexing for 1 min at maximum speed.

Ultrasonic bath for 10 min, vortexing for 1 min at maximum speed.

Ultrasonic bath 10 min, vortexing for 1 min.

Ultrasonic bath for 10 min, vortexing for 1 min.

Adaptation: Ultrasonic bath can be run on ice if thermosensible specialized metabolites are expected in the samples.

Centrifugation: 15,493 *g* (13,000 rpm) 4°C for 10 min for macromolecular compound precipitation. Collect 900 µl of supernatant with positive displacement pipette and dispense it into a 2 mL vial compatible with pH adjustment step. In case of floating particles at the air-liquid interface for some plant samples, use a Pasteur pipette to withdraw the maximum of supernatant volume (at least 600 µl, the lower limit required for pH adjustment step) or program carefully the withdraw needle depth of an automated dispenser to cross the interface and not withdraw particles.

Adjust pH_{apparent} to 6.00 ± 0.02 with NaOD 1 M and/or DCI 1 M solution manually or by means of BTpH titration robot (Bruker, Karlsruhe, Germany).

Store at 4°C for a night if not analyzed immediately, or at -20°C in sealed bag with desiccant. If storage, before opening the vials, let them warm up at room temperature in a desiccator. If needed, centrifuge vials. To a 5-mm NMR tube (e.g. 5 mm 507-pp-7 Wilmad) add 8 µl of TMSP solution and dispense 800 µl of extract. Adjust volume of TMSP solution to extract volume.

At the end of the standardized preparation of NMR instrument and preliminary test on a few selected but representative sample extracts for the selection of 1D pulse sequences with presaturation, the resolution of citrate and malate resonance pattern is checked and if needed an additional EDTA-*d*12 solution volume may be added to each NMR tube of sample set (see Online resource 4).