Seed Storage Reserve Analysis

Harrie van Erp, Guillaume Menard and Peter J. Eastmond*

Department of Plant Biology and Crop Science, Rothamsted Research, Harpenden, UK
*For correspondence: peter.eastmond@rothamsted.ac.uk.

[Abstract] One of the major goals of plant research is to improve crop yield, by for instance increasing seed oil or protein content. Besides this, extensive research is done to change seed fatty acid (FA) composition in order to make vegetable oils more suitable for specific purposes. To determine the effect of genetic changes on seed FA composition, oil, protein and sugar content it's important to use standardised protocols to compare results between different research groups. Here we describe standardised methods for the analysis of seed FA composition, oil, protein and sugar content.

Materials and Reagents

1. Dry oilseed rape (*Brassica napus*) seed samples
2. KCl (Sigma-Aldrich, catalog number: 31248-500G)
3. Methanolic-HCl (Sigma-Aldrich, catalog number: 33050-U)
4. Hexane (Sigma-Aldrich, catalog number: 10549380)
5. Toluene (Sigma-Aldrich, catalog number: 650579)
6. Pentadecanoin (Nu-Chek, catalog number: N-15-A)
7. Heptadecanoin (Nu-Check, catalog number: N-17-A)
8. Fatty acid standard (Supelco 37 component FAME mix) (Sigma-Aldrich, Supelco, catalog number: 47885-U)
9. 6 ml screw cap glass vial (Schott AG, VWR, catalog number: 391-0140).
10. Organic solvent resistant caps for 6 ml screw cap vials (SKS-Science, catalog number: 240409).
11. GC column (BPX70) (SGE Analytical Science, catalog number: 054600)
12. 98% ethanol (Sigma-Aldrich, catalog number: 459844)
13. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: S5881)
14. 80% ethanol (v/v) diluted in distilled water
15. 50% ethanol (v/v) diluted in distilled water
16. 0.1 M NaOH (4 g/L) in distilled water
17. Potassium hydroxide (KOH) (Sigma-Aldrich, catalog number: 484016)
18. HEPES (Sigma-Aldrich, catalog number: H3375)

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19. Magnesium chloride ($MgCl_2$) (Sigma-Aldrich, catalog number: M8266)
20. Adenosine-5'-triphosphate (ATP) (Roche Diagnostics, catalog number: 10 127 531 001)
21. Sucrose (Sigma-Aldrich, catalog number: 84097)
22. Nicotinamide adenine dinucleotide phosphate (NADP) (Roche Diagnostics, catalog number: 10 240 35 4001)
23. Glucose-6-Phosphate Dehydrogenase grade II (G6PDHII) (Roche Diagnostics, catalog number: 10 737 232 001) (80 µl, centrifuge and resuspend pellet in 200 µl of Buffer B)
24. Hexokinase (HK) (Roche Diagnostics, catalog number: 11 426 362 001) (120 µl, centrifuge and resuspend pellet in 200 µl of Buffer B)
25. Phosphoglucose isomerase (PGI) (Roche Diagnostics, catalog number: 10 128 139 001) (60 µl, centrifuge and resuspend pellet in 200 µl of Buffer B)
26. Invertase (INV) (Sigma-Aldrich, catalog number: I4504) (as much as possible to dissolve in 200 µl of Buffer B)
27. 60 mg/ml ATP in distilled water
28. 36 mg/ml NADP in distilled water
29. Buffer A (see Recipes)
30. Buffer B (see Recipes)
31. Reagent mix 1 (see Recipes)

Equipment

1. Gas chromatograph (GC) (Agilent, model: 7890A)
2. MinispecMQ20 (Bruker Corporation)
3. Robotic sample handling system (Rohasys BV)
4. Centrifuges
   For the “Sucrose and protein content determination” procedure: Eppendorf Centrifuge 5415 R (Eppendorf, catalog number: 022621408)
   For the “Determination of seed FA content and composition” procedure: Eppendorf Centrifuge 5810 R (Eppendorf, catalog number: 5811 000.010)
5. Vortex-Genie 2 Shaker (Scientific Industries, Cole-Parmer, catalog number: UY-04724-05)
6. 2 ml Eppendorf tube (Sigma-Aldrich, catalog number: T2795 but not important)
7. Microplate 96 wells (Sigma-Aldrich, catalog number: M4561 but not important)

Procedure

A. Determination of seed FA content and composition

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1. Aliquot 0.4-2 mg of seeds into a 6 ml screw cap glass vial.
2. Add 10 µl pentadecanoin or heptadecanoin standard (1 µg/µl stock in toluene).
   The reason to choose these FAs as standards is that they are not presents in *Arabidopsis* seeds and do not overlap with other fatty acid methyl esters (FAMEs) during GC analysis.
3. Add 1 ml of 1 N methanolic-HCl.
4. Heat for 1.5 h at 80 °C in order to produce FAMEs.
5. Add 200 µl of hexane (100%).
6. Add 1.5 ml of 0.8% KCl and vortex for 10 sec.
   It's important to add the hexane and KCl in this order to avoid artefacts.
7. Centrifuge for 2 min at 2,000 rpm at room temperature in order to induce a phase separation.
8. Pipet of 150 µl of the upper phase and transfer to a GC vial.
9. Run on GC column using the following parameters: 150 °C for 0.1 min, ramp up to 190 °C at 4 °C per min.
10. Analyse data and determine FA content per seed.
11. Retention times of different FAs can be determined by consulting literature or running standards.
    Figure 15 of the *Arabidopsis* book chapter about Acyl-Lipid metabolism shows the result of the GC analysis of *Arabidopsis* seed oil (Li-Beisson *et al.*, 2010).
    On the Sigma-Aldrich website the separation of the Supelco 37 component FAME mix on different GC columns is described (http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Supelco/Bulletin/t196907.pdf).

B. Determination of seed oil and moisture content by low-resolution time domain NMR spectroscopy
1. Construct oil and moisture calibration according to the ISO 10565:1998 standard (http://www.iso.org/iso/catalogue_detail.htm?csnumber=26317). Use nine approximately 0.5 gr seed samples (between 5% and 10% moisture content and between approximately 30% and 55% oil content ($r^2 = 0.99$).
2. Measure oil and moisture content of 0.5 grams of dry seeds.
3. Normalize seed percentage oil content using 9% water content as reference.

C. Extraction for sucrose and protein content determination
1. Take samples of 10-20 intact seeds (minimum) in safe lock 2 ml Eppendorf tube.
2. Add 250 µl of 80% ethanol.
3. Vortex for 30 sec.

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4. Heat samples at 80 °C for 20 min.
5. Centrifuge at 23,000 g for 5 min at room temperature.
6. Transfer the supernatant (S1) in another 2 ml Eppendorf tube on ice.
7. Add 150 µl of 80% ethanol.
8. Vortex for 30 sec.
9. Heat samples at 80 °C for 10 min.
10. Centrifuge at 23,000 *x g* for 5 min at room temperature.
11. Transfer the supernatant (S2) in the corresponding tubes on ice.
12. Add 250 µl of 50% ethanol.
13. Vortex for 30 sec.
14. Heat samples at 80 °C for 10 min.
15. Centrifuge at 23,000 *x g* for 5 min at room temperature.
16. Transfer the supernatant (S3) in the corresponding tubes on ice.
17. Store the tubes containing S1+S2+S3 at -20 °C.
18. Add 400 µl 0.1 M NaOH to the pellet.

D. Sugar and protein content determination (method optimized for microplate 96 wells)
   1. Sugar content determination
      a. In each well dispense:
         50 µl of ethanolic extract (depending of your estimated concentration)
         The protein concentration depends on the species from which the samples are isolated. The amount of protein in the seeds of your species of interest can be checked in the literature.
      b. 160 µl of Reagent mix 1
      c. Read OD at 340 nm for 10 min (until OD stabilizes).
      d. Add 1 µl of HK per well.
      e. Read OD at 340 nm until OD stabilizes (usually 25 min).
      f. Add 1 µl of PGI per well.
      g. Read OD at 340 nm until OD stabilizes (usually 25 min).
      h. Add 1 µl of INV per well.
      i. Read OD at 340 nm until OD stabilizes (usually 1.5 h).
      j. Calculations.
         Calibration curve for sucrose (in duplicate): 200, 100, 50, 25, 12.5, 6.25 µg/ml (in buffer)
         or use the formula: µmol NADPH= ΔOD/(2.85[1]*6.22[2])
         [1]: Pathlength for microplate
         [2]: NADPH molar extinction coefficient

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2. Protein content determination
    a. Heat the samples at 95 °C for 30 min.
    b. Cool down and centrifuge at 23,000 *x g* for 5 min at room temperature.
    c. Use 5-10 µl of the supernatant.
    d. Follow protocol from Thermo Scientific™ Pierce™ BCA™ Protein Assay (23227).
    e. NB: Dilute the standards as appropriate in 0.1 M NaOH.

Recipes

1. Buffer A
   1 M HEPES/KOH
   30 mM $MgCl_2$ (pH 7.0)
2. Buffer B
   0.1 M HEPES/KOH
   3 mM $MgCl_2$ (pH 7.0)
3. Reagent mix 1
   15.5 ml Buffer B
   480 µl ATP
   480 µl NADP
   200 µl G6PDHII

Acknowledgments

This work was supported by the Biotechnology and Biological Sciences Research Council (Institute Strategic Program grant and grant no. BB/E022197/1). The protocol for the determination of seed FA composition was adapted from Browse *et al.* (1986).

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