USDA Hemp Descriptor and Phenotyping Handbook v4

Moisture content

Equipment and supplies

- Forced-draft oven
- Aluminum moisture dishes with tight fitting lids (51 mm D)
- Analytical balance (0.001g accuracy)
- Desiccator cabinet with desiccant
- Drying trays (dimensions to fit on desiccator cabinet shelf)

Procedure:

1. Set oven temperature to 130 °C
2. Label aluminum weighing dish and lid and dry in the oven for about an hour.
3. Store dried aluminum dish in the desiccator cabinet.
4. Weigh aluminum moisture dish (W_t) then add about 10 g whole seeds sample. Record total weight (W_T). Prepare in duplicate.
5. Arrange aluminum dish on dryer tray.
6. Put drying tray in the oven and dry for 3 h.
7. Remove tray from the oven and put the lids on each aluminum dish.
8. Transfer drying tray with dried samples into the desiccator cabinet to cool down.
9. Weigh aluminum dish with dried samples (W_D).
10. Calculate moisture content

$$MC\ (\%) = [(W_T - W_D)/ (W_T - W_t)]\ x\ 100$$

NOTE:
If the seed moisture is > 13%, start drying at 100 °C for 24 – 36 h, them raise oven temperature to 130°C and continue drying for another 3 h.

References:

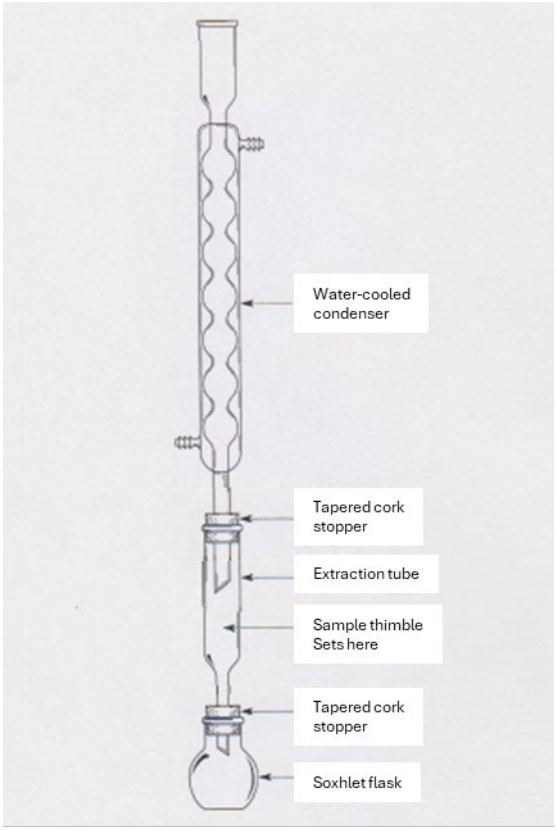
AOCS Official Method Af 2-54 Moisture and Volatile Matter in Flaxseed.
AOCS Official Method Ai 2-75 Moisture and Volatile Matter in Sunflower Seed.
Collison, M.W. (Ed.). (2017). Official Methods and Recommended Practices of the AOCS, 7th Edition. AOCS Press.

Oil content

A. Solvent extraction method

Equipment and supplies

- Analytical balance (0.001g accuracy)
- Mechanical mill - easy to clean, grinds seed uniformly without heating or appreciably change the moisture, volatile matter, or oil content
- Mechanical micro grinder - capable of producing particle size of less than 160 µm
- Cellulose extraction thimbles (25 x 80 mm)
- Absorbent cotton
- Butt-type extraction apparatus (see figure)
    - Water-cooled condenser
    - Extraction tube (32mm O.D. x 125mm, overall length 230mm)
    - Soxhlet flask (200-250 mL)
    - Hot plate with temperature control
- Technical *n*-hexane
- Pumice stone or other anti-bumping granules (dried at 130 °C for 2 h and stored in airtight jar)
- Fume hood
- Rotary evaporator



Butt-type extraction setup.

Procedure

1. Clean seed sample of impurities. Use a magnet to remove fine metal particles, if present.
2. Determine moisture content of the sample. If moisture content is > 10%, dry in an oven set at ≤ 80 °C. to bring moisture to less than 10%.
3. Store sample in airtight jar.
4. Grind at least 50 g of seeds so that no whole seeds remain and 95% passes through the 1 mm sieve. Transfer all ground seeds into an airtight jar. Determine moisture content.
5. Weigh 10 g of ground seed into the extraction thimble and plug with cotton wool. Load the thimble into the extraction tube.
6. Drop a few granules of pumice stone in the Soxhlet flask and weigh to the nearest mg. Pour about 125 mL of n-hexane into the flask.
7. Assemble the extraction apparatus (as shown in the figure) in the fume hood.
8. Start the cooling water to the condenser.
9. Turn on the heater for the Soxhlet flask. Increase the temperature gradually so the n-hexane reflux rate is at least 3 drops per second. Continue extraction for 4 h. Turn off the heater and cool.
10. Remove the thimble from the extraction tube and air-dry in the fume hood to remove the residual solvent.
11. Grind the content of the thimble using a micro grinder to particle size less than 160 µm. Put the finely ground sample back in the thimble. Use cotton wool to collect any sample particles from the grinder and place it in the thimble.
12. If needed, add more solvent in the flask containing the first extract and continue extraction for another 2 h. Allow to cool and air dry the thimble in the fume hood.
13. Repeat steps 11 and 12 and do third extraction for 2 h.
14. Evaporate the solvent in the flask at 80 °C under reduced pressure using a rotary evaporator.
15. Allow the flask to cool in the desiccator for at least an hour and weigh to the nearest mg.
16. Repeat steps 14 and 15.
17. The difference in weight should not exceed 10 mg. If it does, repeat heating for 10 minutes, cooling down and weighing until the difference between two successive weighing does not exceed 10 mg.
18. If a slight increase in weight is observed, take the previous lowest weight.

Calculation:

$$\text{Oil content (\%)} = m_1/m_o \times 100$$

Where:

m_1 = the mass of dried oil (extract) in the flask, g

m_0 = the weight of test sample (step 4), g

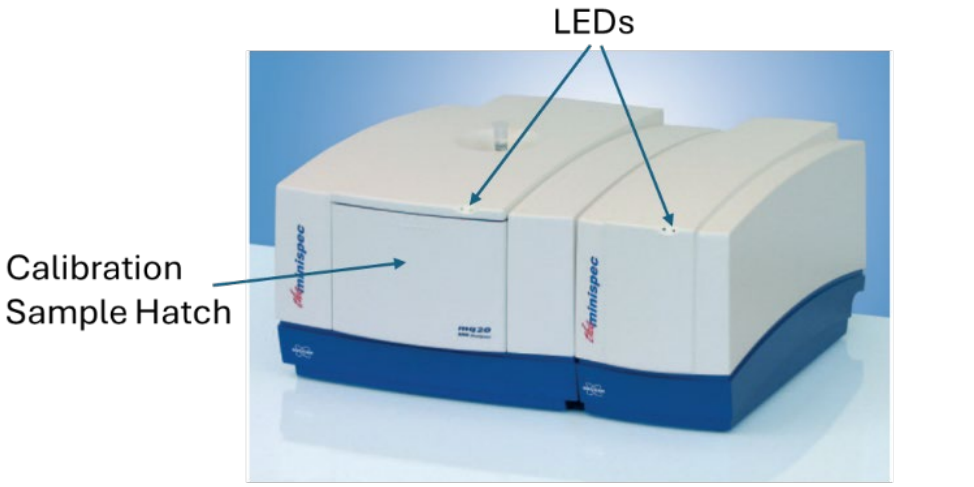
References:

AOCS Official Method Am 2-93 Oil Content in Oilseeds

AOCS Official Method Ai 3-75 Oil content of Sunflower Seeds

Collison, M.W. (Ed.). (2017). Official Methods and Recommended Practices of the AOCS, 7th Edition. AOCS Press.

B. Non-destructive method using Time-Domain Nuclear Magnetic Resonance (TD-NMR) – (The method described below is for the Minispec mq20 (Bruker)



Magnet Unit (left) and Electronic Control Unit (right)

Equipment and Supplies

- the Minispec mq20 (Bruker)
  - mq20 magnet with 25 mm air gap.
  - 20 MHz probe assembly for sample tubes of 18 mm outer diameter.
  - Electronic control unit
  - Minispec Plus software
- Analytical balance (0.00 g)
- Borosilicate tubes -18 x 150 mm
- Dry block heater (set at 40 °C) for 18 mm OD tubes
- Rubber stoppers- size 0

Procedure

1. Turn on the minispec and the PC.
2. Check if the light emitting diodes (LEDs) in front of the instrument are lighting green.

   Electronic control unit (ECU)

   Right LED, connection status indicator
   - ON (green) – PC communication to the ECU is OK
   - OFF – No communication between the ECU and the PC

   Left LED, status indicator
   - ON (green) – The ECU is powered and working
   - OFF – The ECU is not working

   Magnet Unit (MU)

   Right LED, magnet temperature status indicator
   - RED – Pre-heating
   - YELLOW – Transition to normal heating temperature fine adjustment
   - GREEN – Temperature is OK (It will take up to three hours for magnet's temperature to stabilize.)

   Left LED, connection status indicator
   - ON (green) – communication between the MU and ECU is OK
   - OFF – no communication between MU and ECU

3. Click on the minispec NF icon on the display to get into the main menu.
4. Check if the daily check is still valid, otherwise perform the daily check using the check sample tube stored in the compartment/front cover of the magnet. The instrument is ready to use after passing the daily check.
5. Select the method for oil content measurement. If the method has not been established yet, proceed to calibration (below).
6. Prepare samples for analysis
   a. Clean seed sample of impurities. Use a magnet to remove metal particles, if present.
   b. Determine the moisture content of the samples. Moisture content of the samples for TD-NMR analysis should be < 10%.
   c. If moisture content is > 10%, dry in an oven set at ≤ 80 °C. to bring moisture to less than 10%.
   d. Fill the tube with 3 g of samples. Record weight to nearest cg.
   e. Plug tube with rubber stopper.
   f. Place the tubes with samples in the dry block heater for at least 30 min.
   g. Analyze the samples.
7. Choose the method for oil content analysis.
8. Click on measure and follow the prompts to insert samples. The result may be displayed as g of oil or % oil, depending on how the method was set up.

9. Calculate % oil content (for results in g of oil)

$$\% \text{ Oil content} = (\text{g oil}/\text{weight of sample, g}) \times 100$$

Note: Percent oil content will be displayed if sample weights were entered before analysis.

Calibrating the TD-NMR (for oil content measurement only)

1. Prepare 5 borosilicate tubes. Pack the bottom of each borosilicate tube with cotton fiber (~3 mm height).
2. Carefully add aliquots (0.25 g, 1.00 g, 1.175 g, 2.50 g) of hempseed oil directly onto the packed cotton fiber in each tube. Record the exact weight (0.00 g) of oil added to each tube. Do not add oil to tube 1.
3. Plug each tube with a rubber stopper and place in the dry block heater.
4. Open the Minispec Plus menu and perform the daily check.
5. Click on the calibrate button to enter the calibration menu.
6. Select create a new calibration and give a descriptive name for the method.
7. Select the corresponding Bruker pre-defined calibration method that suits your sample type or select the new method you intend to use.
8. Verify and adjust important acquisition settings such as the number of scans and recycle delay times. Default settings are shown below.

Parameters	
Scans	16
Rd [s]	2.00
Gain [dB]	50
Dig. Bw [Hz]	781
Det. Mode	magnitude
Magnitude Mode	DIODE
Dummy Shots	0
Gradient Unit	None
Settings	
90 Pls. Len. [us]	15.92
180 Pls. Len.	31.52
Det Angle.B [deg]	355
H Offs. [Steps]	1108
NMR Freq1 [MHz]	19.950000
Pulse Atten [dB]	19
Instr. Gain [db]	45
Dead Time [ms]	0.0336
Homog. Limit [ms]	0.20
Instr. Rd [s]	2.00
Stat Grad X [%]	0.00

Stat Grad Y [%]	0.00
Stat Grad Z [%]	0.00
SFC	no

9. Start the calibration and perform gain tuning with the sample having the highest amount of oil to optimize signal acquisition.
10. Continue measuring each calibration sample sequentially following the prompts in measuring one sample after another.
11. Review calibration data. Add or remove samples as necessary to improve calibration fit and accuracy (≥0.990).
12. Sign and save the calibration.
13. Exit the calibration menu.

Reference:

AOCS Official Method Ak 3-94 Oil Content of Oilseeds by Nuclear Magnetic Resonance
Collison, M.W. (ed.). (2017). Official Methods and Recommended Practices of the AOCS, 7th Edition. AOCS Press.

Crude protein content

A. Kjeldahl Method

Equipment

- Digestion block with temperature control, digestion tube rack, heat shield, and fume exhaust manifold connected to a water aspirator in a hooded sink. (Tecator Digestion System 20, 1015 Digestor, Foss North America or equivalent)
- Digestion tubes (250 mL)
- Distillation unit to accept 250 mL digestion tubes (Tecator 2200, or equivalent)
- Titration flask (500 mL)
- Weighing paper (low N)
- Balance (0.000g)
- Adjustable volume pipetting dispenser (≤25 mL) attached to 2.4 L acid bottle
- Safety equipment (cotton lab coat, safety glasses, chemical resistant gloves, gloves for handling hot tubes)
- Titration burette – 25 mL
- Magnetic stirrer and stir bar

Reagents

- Sulfuric acid - 95-98% H_2SO_4, reagent grade
- Catalyst – 7.0g K_2SO_4 + 0.8 g $CuSO_4$ (commercially available in tablet form, 3.5 K_2SO_4 + 0.4 g $CuSO_4$ per tablet)
- Sodium hydroxide – 40% w/w NaOH, low N (≤5µg N/g)
- Kjeldahl boric acid solution with indicators (bromocresol green/methyl red) – 4% (commercially available)
- Hydrochloric acid standard solution - 0.10M (commercially available)
- Reference standards
    - Acetamilide (Baker A068-05)
    - Tryptophan (Sigma T 8659)
    - $(NH_4)_2SO_4$ (100% assay)
- Sucrose (N-free)

Procedure

Preparation of sample

1. Grind the seed sample using a knife mill (recommended for seeds with high oil content) to 0.7-1 mm particle size.
2. Transfer ground sample into a capped bottle.

3. Determine the moisture content of starting seed sample and ground sample.

Digestion

4. Turn on the digestion block and heat to 420 °C.
5. Weigh 1 g of ground sample onto a tared low N weighing paper. Fold paper around material and drop into the Kjeldahl tube. Record the exact weight of the sample to the nearest mg.
6. Add into the Kjeldahl tube 2 tablets of catalyst and 15 mL of sulfuric acid using the pipetting dispenser.
7. Place tube in the tube rack.
8. Attach heat side shield to tube racks and fume manifold on tubes.
9. Turn water aspirator on completely.
10. Place rack in preheated digestion block
11. After 10 min, turn the water aspirator down until acid fumes are just contained within the exhaust hood. A condensation zone should be maintained within the tube. After bulk of sulfur oxide fumes are produced during the initial stages of digestion, reduce vacuum source to prevent loss of H_2SO_4. Continue digestion for 50 min. Total digestion time is approximately 60 min.
12. Turn the digestor off and remove the digestion rack with exhaust still in place and allow it to cool for 10-20 min. Remove the manifold and shut off the aspirator once fuming has stopped.
13. Remove the heat shields and let the tubes cool.
14. Carefully add a few mL of deionized water to each tube up to 80 mL. This can be omitted if the distillation unit uses steam to equilibrate the digested sample.

Distillation

15. Fill the distillation unit's alkali tank with 40% NaOH and adjust volume dispensed to 50 mL.
16. Attach the digestion tube to the distillation unit.
17. Fill Erlenmeyer flask with 30 mL of the 4% H_3BO_3 solution with indicator and place on receiving platform of the distillation unit. Immerse the tube from the condenser below the surface of the H_3BO_3 solution.
18. Steam distill until ≥150 mL distillate is collected (≥180 mL total volume).

Titration

19. Remove flask and add stir bar and set flask on the magnetic stirrer.
20. Fill the burette with standard 0.100M HCl. Record the starting volume.

21. Add titrant slowly to the sample and titrate to violet end point (just before the solution turns pink). Record the volume of HCl used to the nearest 0.05 mL.

Verification of Nitrogen Recovery

22. Nitrogen loss: Use 12 g $(NH_4)_2SO_4$ and 0.67 g sucrose per flask and perform distillation as described above. Recoveries must be ≥99%.
23. Distillation and titration efficiency: Distill 0.12 g $(NH_4)_2SO_4$. Recoveries must be ≥99.5%.
24. Digestion efficiency: Use 0.3 g acetamide or 0.18 g tryptophan, with 0.67 g sucrose per flask. Digest and distill as described above. Recoveries must be ≥98%.

Calculations

$$\text{Kjeldahl N (\%)} = [(\text{Vs} - \text{Vb}) \text{ x M x } 14.01]/(\text{W x } 10)$$

Crude protein (%) = Kjeldahl %N x F

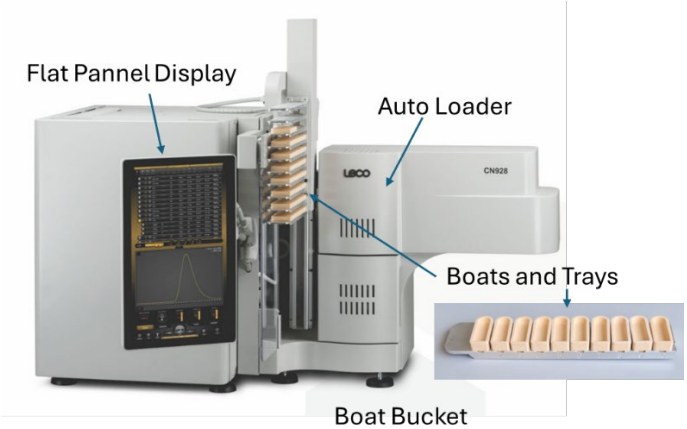
Where:

Vs = volume (mL) of standardized acid used to titrate a test
Vb = volume (mL) of standardized acid used to titrate reagent blank
M = molarity of standard HCl
14.01 =atomic weight of N
W = weight (g) of test portion
10 = factor to convert mg/g to percent
F = factor to convert N to crude protein (= 6.25 for oilseeds)

Reference

AOAC Official Method 2001.11 – Protein (Crude) in Animal Feed, Forage (Plant Tissue), Grain, and Oilseeds: Block Digestion Method Using Copper Catalyst and Steam Distillation into Boric Acid. Latimer Jr., G. (ed.), 2023. Official Methods of Analysis of AOAC International (22nd Ed). https://doi.org/10.1093/9780197610145.001.0001

B. Combustion Method (Dumas N)



Equipment

- LECO FP928 Macro N analyzer with Cornerstone® software
- Unglazed ceramic combustion boats (LECO 528-203)
- Analytical balance (0.0001 g)
- Carrier gas -Helium. 99.99% pure, 25 psi %
- Combustion gas – Oxygen, 99.99% pure, 25 psi ± 10%
- Pneumatic gas – Compressed air, oil and water free, 40 psi ± 10%
- EDTA LCRM (LECO 502-896-250)

Procedure

1. Prepare instrument for operation as outlined in the operator's instruction manual. Select the method for the analysis.

Method summary

Parameters	
Gas	Helium
Furnace temperature	1,100 °C
Ramp rate	12 °C/min
Dehydration time	0 s
Nominal sample mass	1.000 g
Purge cycles	3
Ballast equilibrate time	10 s
Ballast not filled timeout	300 s
Aliquot loop fill pressure drop	200 mmHg
Aliquot loop equilibrate time	4 s
Dose loop size	3 cm^3 (small)

Burn Steps		
Step 1	Lance flow	No
	Furnace flow	Yes
	Time	5 s
Step 2	Lance flow	Yes
	Furnace flow	Yes
	Time	5 s
Step 3	Lance flow	Yes
	Furnace flow	No

2. Condition the System.
   a. Select five or more blank replicates in the Login screen (ceramic combustion boat is not required).
   b. Initiate the analysis sequence.
3. Determine blank.
   a. Select five or more blank replicates in the Login screen.
   b. Place ceramic combustion boats in the appropriate positions in the autoloader.
   c. Initiate the analysis sequence.
   d. Set the blank following the procedure outlined in the operator's instruction manual.
   Note: The standard deviation of the last five blanks should be less than or equal to 0.001% (10 ppm) when utilizing Helium as a carrier gas. Additional blanks beyond the recommended five may be required to achieve the recommended precision.
4. Calibrate/Drift Correct.
   a. Select the desired number of Calibration/Drift replicates in the Login screen (minimum of five).
   b. Weigh an appropriate mass of suitable reference material into a 528-203 Ceramic Combustion Boat.
   c. Enter sample mass and identification into the Login screen.
   d. Transfer the ceramic combustion boat containing the reference material to the appropriate position in the autoloader.
   e. Perform steps 4b through 4d a minimum five times for each calibration/drift material used.
   f. Initiate the analysis sequence.
   g. Calibrate or Drift Correct the instrument following the procedure outlined in the operator's instruction manual.

h. Verify the calibration by analyzing an appropriate mass of another suitable reference material and confirm that the results are within the acceptable tolerance range.

Note: Typically, the LECO FP928 can be calibrated utilizing several replicates of a single mass range (nominal 0.75 g) of EDTA utilizing a single standard calibration (linear, force through origin calibration). This is a cost effective and simple process. The calibration can be verified by analyzing different compounds such as nicotinic acid (0.15 to 0.35 g), or phenylalanine (0.15 to 0.5 g). A multi-point calibration (fractional masses or multiple calibration samples) may be used to calibrate if desired.

5. Analyze Samples.
   a. Select the desired number of sample replicates in the Login screen.
   b. Weigh approximately 1.0 g of the sample material into a 528-203 Ceramic Combustion Boat.
   c. Enter sample mass and identification information into the Login screen.
   d. Transfer the ceramic combustion boat containing the sample to the appropriate position in the autoloader.
   e. Perform steps 5b through 5d for each sample to be analyzed.
   f. Initiate the analysis sequence.

Note: If soot (carbon black) is noticed in the primary filter (steel wool filter), reduce the sample mass to prevent soot build-up in this filter. Soot can be produced when larger masses of high fat samples are analyzed. Use 0.400 g for hempseeds.

References

LECO Application Note (Instrument: FP928), 2022. Determination of nitrogen/protein in feed, grains, and pet food. LECO Corporation, Saint Joseph, Michigan USA

Oil fatty acid composition analysis

Equipment

- Handheld homogenizer drive (CAT X120 or equivalent)
- Homoginizer (CAT T10-V or equivalent)
- 20 mL scintillation vials with cap
- Dry block heater for scintillation vials
- GC vial 12 x 32 mm (National C4000-1W)
- Screw tread cap for GC vials (National C5000-51B)
- Adjustable volume bottle top dispensers for dispensing sodium methoxide, hexane, and saturated NaCl solution
- Glass transfer pipettes
- GC equipped with flame-ionization detector (FID) and autosampler/injector
- GC column – 30 m X 0.25 mm i.d. X 0.20 µm thickness (Zebron Capillary GC Colum ZB-FAME, Part No. 7HG-G033-10, phenomere)

Reagents

- 0.25M sodium methoxide in methanol
- Hexane (high purity gas chromatographic grade)
- NaCl, reagent grade, saturated. Dissolve 36 g NaCl in 1 L distilled water
- Helium (carrier gas) – 99.995% or better, gas chromatography quality
- Nitrogen (make-up gas) – 99.995% min, chromatography grade
- FAME standards - a mix of saturated and unsaturated fatty acid methyl esters (FAME) C8-C30 and 18:1, 18:2, 18:3, 20:1, 22:1(Nu-Check Prep)

Preparing FAME from seeds

1. Coarse grind seeds using a coffee grinder.
2. Transfer 1.0 g ground sample into a 20 mL scintillation vial.
3. Add 5 mL 0.25M sodium methoxide in methanol solution.
4. Blend using a homogenizer for ~10 sec. Rinse the homogenizer with methanol.
5. Cap the vial and incubate in the dry heating block (60 ^{o}C) for 30 minutes.
6. Remove vial from heating block and cool to room temperature.
7. Add 5 mL of hexane followed by 5 mL of saturated sodium chloride solution.
8. Cap the vial and mix thoroughly. Let stand to allow layers to separate.
9. Draw ~0.25 mL from the top (hexane) layer and transfer into a GC target vial.
10. Add 2 mL hexane to the target vial and then cap.
11. Prepare test sample in duplicate.

Prepare reference standard mix

12. Transfer the contents of one Nu-Chek Pre vial FAME standard mix to a clean 100 mL volumetric flask. Use hexane to rinse remaining FAME in the vial and add to the volumetric flask.
13. Fill volumetric flask with hexane to the fill line. Place a stopper and mix.
14. Remove the stopper and heat slightly using the steam bath to dissolve solid methyl esters. Ensure the final volume is at the line, add more if hexane if needed.
15. Transfer ~1.5-2 ml of standard mix solution into crimp cap GC vials using a glass transfer pipette. Crimp the vials with the lids, making sure that they seal tightly. Should make between 50 to 65 vials.
16. Label vials with "STD MIX" and "DATE" and store in the freezer (-20℃).

FAME Analysis

17. GC operating conditions
    a. Temperature program
        - Initial 160 °C, hold 4 min, program ramp 160 - 195 °C at 7.5 °C/min, 195 - 280 °C with 5 min hold
        - Injector and detector temperature - 250°C.
    b. Septum purge of 4.0 mL/min
    c. Helium flow rate to the column - 1.2 mL/min at 138 kPa (20 psi)
    d. Injection volume – 2.0 µL
    e. Split ratio - 100:1.
18. Run blank. Check the chromatogram of the blank vial for any interference (contamination and background noise) that may introduce errors in the test sample measurements.
19. Run the standard mix. Check the chromatogram of reference standards to make sure the FAMEs are accounted for.
20. Run test samples. Identify peaks in test samples by comparing their retention times with reference standards.
21. Calculate the fatty acid distribution as percent of total peak areas. Exclude unidentified peaks in the summation of peak areas unless they have been confirmed to be fatty acids.

$$\text{Area \% of the component i expressed as methyl ester} = (A_i/\sum A)/100$$

Where, A_i = peak area of component i

$\sum A$ = sum of areas of all identified peaks

References

AOCS Official Method Ce 1a-13 (revised 2017), Fatty acids in edible oils and fats by capillary GLC. Collison, M.W. (Ed.). (2017). Official Methods and Recommended Practices of the AOCS, 7th Edition. AOCS Press.

Isbell, T.A., Cermak, S.C., 2015. Registration of Katelyn *Thlaspi arvense* L. (Pennycress) with improved nondormant traits. J. Plant Regist. 9:212-215. https://doi.org/10.3198/jpr2014.08.0053crg