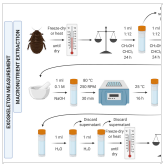

May 10, 2021

MEDI: Macronutrient Extraction and Determination from Invertebrates

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1 Works for me dx.doi.org/10.17504/protocols.io.buj9nur6

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ABSTRACT

Macronutrients, comprising carbohydrates, proteins and lipids, underpin many ecological processes, but their quantification in ecological studies is often inaccurate and laborious, requiring large investments of time and bulk samples, which make individual-level studies impossible. This is a protocol for the direct, rapid and relatively low-cost determination of macronutrient content from single small macroinvertebrates.

Macronutrients are extracted by a sequential process of soaking in 1:12 chloroform:methanol solution to remove lipid and then solubilising tissue in 0.1 M NaOH. Proteins, carbohydrates and lipids were determined by colorimetric assays from the same individual specimens.

Macronutrient Extraction and Determination from Invertebrates (MEDI) can directly and rapidly determine macronutrient content in tiny (dry mass ~3 mg) and much larger individual invertebrates. Using MEDI, the total macronutrient content of over 50 macroinvertebrates can be determined within around 3 days of collection at a cost of ~$1.35 per sample.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Cuff, JP, Wilder, SM, Tercel, MPTG, et al. MEDI: Macronutrient Extraction and Determination from invertebrates, a rapid, cheap and streamlined protocol. Methods Ecol Evol. 2021; 12: 593– 601. https://doi.org/10.1111/2041-210X.13551

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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KEYWORDS

macronutrient, nutritional, nutrient, invertebrate, insect, colorimetric assay, entomology, entomological, exoskeleton, lipid, protein, carbohydrate

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IMAGE ATTRIBUTION

Figures created with Biorender.com.

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GUIDELINES

This protocol is based around that presented in the original manuscript published in Methods in Ecology & Evolution. There are several steps that could be adjusted according to the specific tissue and sample types

MATERIALS TEXT

Equipment:
Plate spectrophotometer
Sensitive weighing scales (ideally to 0.01 mg)
Plate shaker/vortex (ideally with simultaneous heating functionality)
Pipettes (ideally including 8-well multi-channels)
Ventilated fume hood
Tissue lysis equipment (pestle and mortar, or bead-beating equipment with reusable steel beads)
Forceps (for cracking invertebrate exoskeletons)

Consumables:
Flat-bottom 96-well colorimetric assay plates for spectrophotometry
Sample tubes (1.5-2 ml, ideally screw-top)
Pipette tips
Chloroform
Methanol
0.1 M NaOH
Water (ideally polished)
Lipid standard (e.g. analytical lard oil)
Carbohydrate standard (e.g. analytical corn starch)
Protein standard (e.g. bovine serum albumin)
Vanillin
85% phosphoric acid
Anthrone
Concentrated sulfuric acid
Lowry reagent and 1X Folin-Ciocalteu reagent (or alternative appropriate protein assay kit; usually sold together)

SAFETY WARNINGS

Please read the relevant COSHH and safety documentation for the equipment necessary. Particular care must be taken with the concentrated acids, chloroform and sodium hydroxide included in the protocol.

BEFORE STARTING

Please read the full protocol before starting in order to decide which section are relevant to your study and how the overlapping aspects (if you decide to overlap them) should be followed.

Welcome to MEDI!

1 Welcome to Macronutrient Extraction and Determination from Invertebrates! Here's an overview of the extraction protocol:

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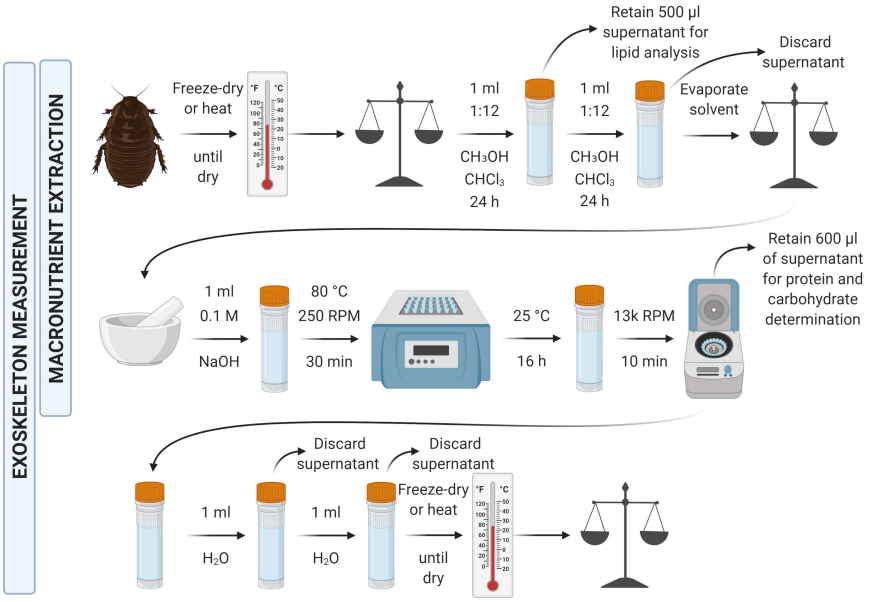

The extraction process for MEDI.

Jordan P. Cuff, Shawn M. Wilder, Maximillian P. T. G. Tercel, Rhiannon Hunt, Somoye Oluwaseun, Paige S. Morley, Rafael A. Badell-Grau, Ian P. Vaughan, James R. Bell, Pablo Orozco-terWengel, William O. C. Symondson, Carsten T. Müller (2021). MEDI: Macronutrient Extraction and Determination from invertebrates, a rapid, cheap and streamlined protocol. Methods in Ecology and Evolution.
https://doi.org/10.1111/2041-210X.13551

There are several overnight incubation steps, so don't be alarmed by some of the long procedure times!

Collection and preparation of materials 1d 0h 5m

2 Collect invertebrates and kill them, ideally by freezing. 4h 5m

If working with fluid-preserved specimens (e.g. alcohol-fixed), transfer all material including preserving agent into a suitable tube (e.g. 1.5 ml microcentrifuge tube or 2 ml screw-top collection tube) and evaporate the preservative (if possible/appropriate) to ensure all solubilised material is accessible for downstream analysis. This can be achieved during the drying described in step 2.

3 Desiccate the invertebrate specimens via freeze-drying or heat-drying until completely dry. 1d

Heat-drying can be carried out in a 60 °C oven for 24-48 hours, or a shorter time period if using a heated centrifuge with a vacuum pump.

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4 Weigh the dried invertebrate using appropriately sensitive scales, recording the mass. 5m

If the invertebrate was fluid-preserved and the preserving agent has been evaporated into the sample tube, ensure you measure the mass of the tube and any precipitated matter it contains as this will be important later, particularly if using gravimetric estimation of lipid content.

If the invertebrate mass is greater than 10 mg, a gravimetric measurement of lipid (detailed below) will likely be more accurate, and following lipid analysis it will be necessary to sub-sample the remaining tissue. If the invertebrate is less than 10 mg, proceed with colorimetric assays without sub-sampling.

Determination of exoskeletal mass 3d 10h 38m

5 For the greatest accuracy, exoskeletal mass determination should be carried out in parallel to the macronutrient analyses with different specimens, but it is possible to streamline these together if necessary. If you do wish to streamline them, the protocol for exoskeletal mass determination largely follows the steps for the macronutrient determination, so attention will be drawn to the overlapping aspects. In order to streamline these processes, exoskeletal determination should take place after extraction of samples for lipid, and carbohydrate and protein determination. When lysing the tissue for protein and carbohydrate determination, the exoskeleton should instead simply be cracked to facilitate penetration of the tissue by the NaOH without breaking apart the exoskeleton itself.

6 1d

Add 1 ml of 1:12 chloroform:methanol to each sample tube and leave the tube containing the specimen and chloroform:methanol at room temperature for 24 hours.

This can be designed to overlap with STEP 17 of "Determination of lipid content".

If working with very large samples (e.g. >50 mg), it is recommended to add ~5X the body volume of the invertebrate.

Different ratios of chloroform and methanol can be used (e.g. 2:1, 1:9), including pure chloroform. It is advised to consistently use the same ratio.

7 1d

Discard the supernatant, avoiding the removal of any tissue, add another 1 ml of 1:12 chloroform:methanol to each sample tube and leave the tube containing the specimen and chloroform:methanol at room temperature for 24 hours.

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This can be designed to overlap with STEP 28 of "Determination of protein and carbohydrate content". The removal of residual lipids is necessary for the accurate analysis of protein content given the inhibition of protein assays by lipids.

8 Discard the supernatant, avoiding the removal of any tissue, and evaporate any remaining residual solvent. 15m

This can be designed to overlap with STEP 29 of "Determination of protein and carbohydrate content".

9 Add 0.1 M NaOH to the sample tube, aiming for 5-10X the body volume of the invertebrate. Lightly crack the exoskeleton to expose the inner tissues, and incubate at 80°C for 2 h. 2h 2m

This can be designed to overlap with STEP 31 of "Determination of protein and carbohydrate content", but with an undefined volume of solvent (rather than 1 ml), cracking of the exoskeleton rather than lysis, and increased incubation time.

10 16h

Allow the sample to soak overnight at room temperature.

This can be designed to overlap with STEP 32 of "Determination of protein and carbohydrate content".

11 Discard the supernatant, avoiding the removal of any tissue. Add a further round of 0.1 M NaOH (the same volume) to the sample tube, and incubate at 80°C for 2 h. 2m

Lightly centrifuge to move the exoskeletal fragments to the bottom of the tube if necessary.

12 16h

Allow the sample to soak overnight at room temperature.

13 Discard the supernatant, avoiding the removal of any tissue. Add water to the tube (the same volume as with NaOH) and remove (without removing any exoskeletal mass) to wash away any remaining NaOH. 2m

14 Repeat the water wash in the previous step so that it has been carried out twice in total. 1m

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15 Remove the water (without removing any exoskeletal mass) and evaporate any remaining water. 15m

16 Weigh the remaining exoskeleton in the tube and subtract the mass of the tube to calculate the exoskeletal mass. 1m

Determination of lipid content 1d 1h 13m

17

Add 1 ml of 1:12 chloroform:methanol to each sample tube and leave the tube containing the specimen and chloroform:methanol at room temperature for 24 hours.

If working with very small samples (e.g. <0.5 mg dry mass), it is recommended to use 0.5 ml instead to increase the downstream concentrate of analyte. This must be factored into the subsequent calculations of lipid content.

If working with very large samples (e.g. >50 mg), it is recommended to add ~5X the body volume of the invertebrate, the volume of which must be recorded and factored into the subsequent calculations of lipid content.

18 Remove 500 µl of chloroform:methanol solution and keep it in a separate tube for lipid analysis. Unless the invertebrate dry body mass is >10 mg, the remaining solution in the original tube is taken forward for determination of protein and carbohydrate content (STEP 31), ideally with immediacy given additional overnight steps. If the body mass is >10 mg, the sub-steps below detail gravimetric lipid determination. 1m

Ensure the tube containing the lipids is air tight to avoid evaporation, which will affect downstream concentration values. Importantly, the tube must also be suitable for the storage of chloroform (i.e. won't dissolve) for which glass is ideal. To avoid potential removal of tube labels written in pen, paper with pencil writing on it can be left in the solution within the tube so long as risk of cross-contamination with lipids is mitigated.

18.1 1d

From the original tube containing the remaining chloroform:methanol solution and the invertebrate tissue, discard the remaining supernatant (having already removed 500 µl for lipid analysis) avoiding the removal of any tissue. Add another of the same volume of 1:12 chloroform:methanol as in STEP 17 to each sample tube and leave the tube containing the specimen and chloroform:methanol at room temperature for 24 hours.

18.2 Discard the supernatant, avoiding the removal of any tissue, and evaporate any remaining residual solvent. 15m

18.3 Weigh the remaining body mass and subtract it from the original dry mass to determine total lipid mass gravimetrically. 1m

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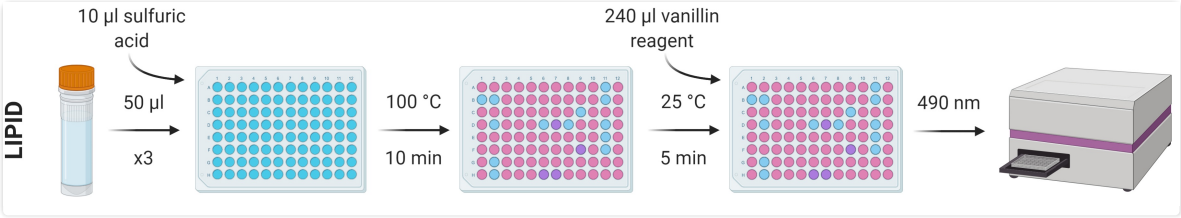
19 If possible, estimate the lipid content of the invertebrates and dilute accordingly to take forward a sub-sample of 0.5-1.75 mg/ml.

20 The lipid content of the 500 µl of chloroform:methanol solution removed during STEP 18 can be determined using the sulfo-phospho-vanillin method.



21 Prepare a stock standard dilution series using a suitable analogue (e.g. analytical lard oil) of known concentration diluted with 1:12 chloroform:methanol. A dilution series of 0-2 mg/ml in nine increments (0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml) should suitably cover a good range of concentrations, but this can be adjusted accordingly. 15m

22 Make up the vanillin reagent using the following amounts (which should account for reagent overage) per sample/standard repeat (so thrice per sample/standard if running triplicates, as advised):

A	B
Reagent	Amount
Vanillin	330 mg
Hot water	220 µl
85% phosphoric acid	55 µl

23 From each standard and sample, put three repeats of 50 µl into a flat-bottomed 96-well plate and heat at 100 °C in a ventilated hood until all solvent has evaporated (leaving just the lipid residue; ~10 min). 12m

24 Add 10 µl concentrated sulfuric acid to the lipid residues and vortex/mix before incubating again at 100 °C for 10 min. 13m

25 Allow the samples to cool to room temperature and add 240 µl vanillin reagent to each well, vortexing/mixing for homogenous colouration. 3m

26 After 5 min, transfer 200 µl of each well into a new plate. 8m

This is mostly in case the plate has warped at all during the heating stage, but also to remove any potential for particulate matter that may have precipitated during the initial evaporation.

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27 Measure absorbance at 490 nm using a spectrophotometer. 5m

Determination of protein and carbohydrate content 1d 18h 37m

28 1d

From the original tube containing the remaining chloroform:methanol solution and the invertebrate tissue, discard the remaining supernatant (having already removed 500 µl for lipid analysis) avoiding the removal of any tissue. Add another 1 ml of 1:12 chloroform:methanol to each sample tube and leave the tube containing the specimen and chloroform:methanol at room temperature for 24 hours.

This additional chloroform treatment is purposed to remove any residual lipids which can interfere with the downstream analyses.

29 Discard the supernatant, avoiding the removal of any tissue, and evaporate any remaining residual solvent. 15m

30 Lyse the tissue and ensure it is mixed/homogenous. Lysis can be carried out using a pestle and mortar, or bead-beating method, ensuring all available tissue is taken forward (unless the dry body mass exceeds 10 mg, in which case follow the below sub-step). 5m

If using a bead-beating method (e.g. Qiagen Tissuelyser II at 30 Hz for 8 min), it may be easier to do so in a liquid matrix, so you can do this after the initial addition of NaOH in the next step. Care must be taken to avoid contact with NaOH when doing so.

30.1 If working with invertebrates with a dry body mass >10 mg, lyse/homogenise the body and take a 3-7 mg subsample forward for the subsequent steps. 2m

Take note of the mass of tissue taken forward. The subsequent absorbance readings will only represent this mass of tissue and must be multiplied to calculate the total protein/carbohydrate content of the whole specimen.

With 3-7 mg of dry tissue, dilutions will still likely be necessary downstream (detailed in STEP 35). This must also be accounted for in any calculations of total protein/carbohydrate content (i.e. the values will be for 3-7 mg of the specimen rather than its full mass).

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31 Add 1 ml 0.1 M NaOH to the sample tube. Incubate at 80°C for 30 min in a themo-shaker to ensure mixture of the lysed material with the NaOH. 32m

If working with very small samples (e.g. <0.5 mg dry mass), it is recommended to use 0.5 ml instead to increase the downstream concentrate of analyte. This must be factored into the subsequent calculations of macronutrient content. Importantly, less than 0.6 ml can then be taken in STEP 34, but 0.4 ml should still be sufficient.

32 16h

Allow the sample to soak overnight at room temperature.

This overnight step can be left out if ensuring the tissue is appropriately lysed, for example by including sonication in STEP 31 and mixing halfway through.

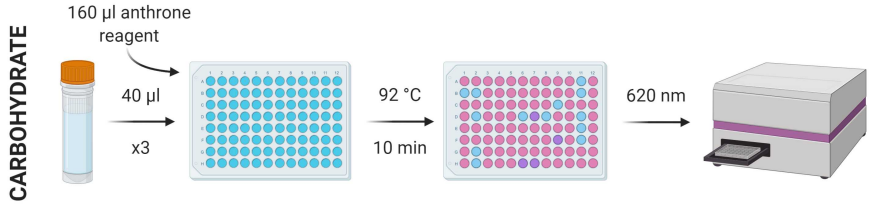
33 Centrifuge the sample for 10 min at 13,000 rpm. 11m

34 Take 600 µl of the supernatant into a separate tube for taking forward for protein and carbohydrate determination. 1m

35 If possible, estimate the protein and carbohydrate content of the invertebrates and dilute accordingly for each assay to take forward 0.5-1.75 mg/ml.

If using 3-7 mg of dry tissue, as per STEP 30.1, a 1 in 5 dilution will likely be appropriate (i.e. 100 µl of sample with 400 µl NaOH). Remember to factor any dilutions into the final calculation of protein.

36 The carbohydrate content of the 600 µl of NaOH solution removed during STEP 34 can be determined using the anthrone method.



37 Prepare a stock standard dilution series using a suitable analogue (e.g. analytical corn starch) of known concentration diluted with polished water. A dilution series of 0-2 mg/ml in nine increments (0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml) should suitably cover a good range of concentrations, but this can be adjusted accordingly. 5m

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38 Make up the anthrone reagent using the following amounts (which should account for reagent overage) per sample/standard repeat (so thrice per sample/standard if running triplicates, as advised): 2m

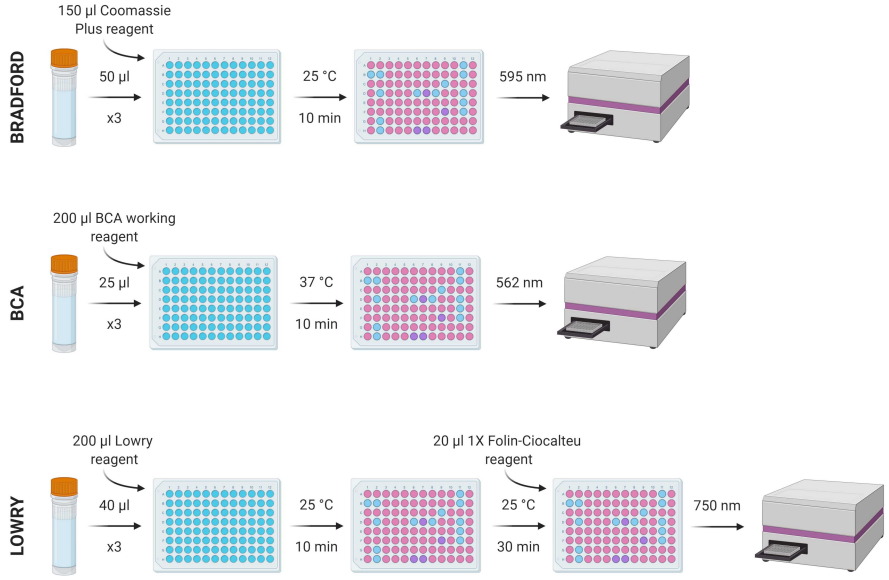
A	B
Reagent	Amount
Anthrone	185 mg
Concentrated sulfuric acid	185 µl

39 From each standard and sample, put three repeats of 40 µl into a flat-bottomed 96-well plate, add 160 µl anthrone reagent to each and vortex/mix. 3m

40 Incubate the plate at 92 °C in a ventilated hood for 10 min. 10m

41 Cool the plate to room temperature and measure absorbance at 620 nm using a spectrophotometer. 15m

42 The protein content of the 600 µl of NaOH solution removed during STEP 34 can be determined using one of several colorimetric protein methods. The three highlighted in the MEDI manuscript (the benefits and drawbacks also discussed therein) are summarised in the image below. This protocol will describe the protocol for the Lowry assay. The reagents involved in each of these assays are widely commercially available.



43 Prepare a stock standard dilution series using a suitable analogue (e.g. bovine serum albumin) of known concentration diluted with polished water. A dilution series of 0-2 mg/ml in nine increments (0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml) should suitably cover a good range of concentrations, but this can be adjusted accordingly. 5m

Most protein assay kits will allow values to be converted for different standards using standard conversion factors

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since different standards will differentially affect the results of each assay.

44 From each standard and sample, put three repeats of 40 µl into a flat-bottomed 96-well plate, add 200 µl of Modified Lowry Reagent to each and vortex/mix. 3m

45 Incubate at room temperature (~20-25 °C) for 10 min. 10m

46 Add 20 µl of 1X (1N) Folin-Ciocalteu reagent to each well and vortex/mix. 3m

47 Incubate at room temperature (~20-25 °C) for 30 min. 30m

48 Measure absorbance at 750 nm using a spectrophotometer. 5m

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