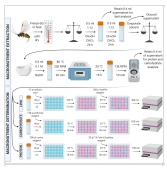


Sep 17, 2021

Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates)

Forked from MEDI: Macronutrient Extraction and Determination from Invertebrates

Jordan Cuff[1]

[1]Cardiff University

dx.doi.org/10.17504/protocols.io.bw5hpg36

Jordan Cuff

Jordan Cuff

DISCLAIMER

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 <1 mg). 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.

DOI

dx.doi.org/10.17504/protocols.io.bw5hpg36

PROTOCOL CITATION

Jordan Cuff 2021. Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates). **protocols.io**
https://dx.doi.org/10.17504/protocols.io.bw5hpg36

FUNDERS ACKNOWLEDGEMENT

BBSRC
Grant ID: BB/M009122/1

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

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

FORK NOTE

This protocol is a further micro-scaled version of the original MEDI protocol.

FORK FROM

Forked from MEDI: Macronutrient Extraction and Determination from Invertebrates, Jordan Cuff

KEYWORDS

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

IMAGE ATTRIBUTION

Figures created with Biorender.com.

CREATED

Aug 04, 2021

LAST MODIFIED

Sep 17, 2021

PROTOCOL INTEGER ID

52105

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.

Citation: Jordan Cuff (09/17/2021). Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates). https://dx.doi.org/10.17504/protocols.io.bw5hpg36

MATERIALS TEXT

Equipment:
Plate spectrophotometer
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)

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)

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. Prolonged incubations are included, which should be vigilantly monitored.

DISCLAIMER:

BEFORE STARTING

Please read the full protocol before starting and ensure that you have all the necessary equipment/reagents. Particular attention must be paid to the COSHH and safety documentation relating to the reagents. The original MEDI protocol is recommended for samples with dry masses greater than 1 mg. For samples which are borderline, consider using the original protocol before this adaptation; it will likely produce more accurate results.

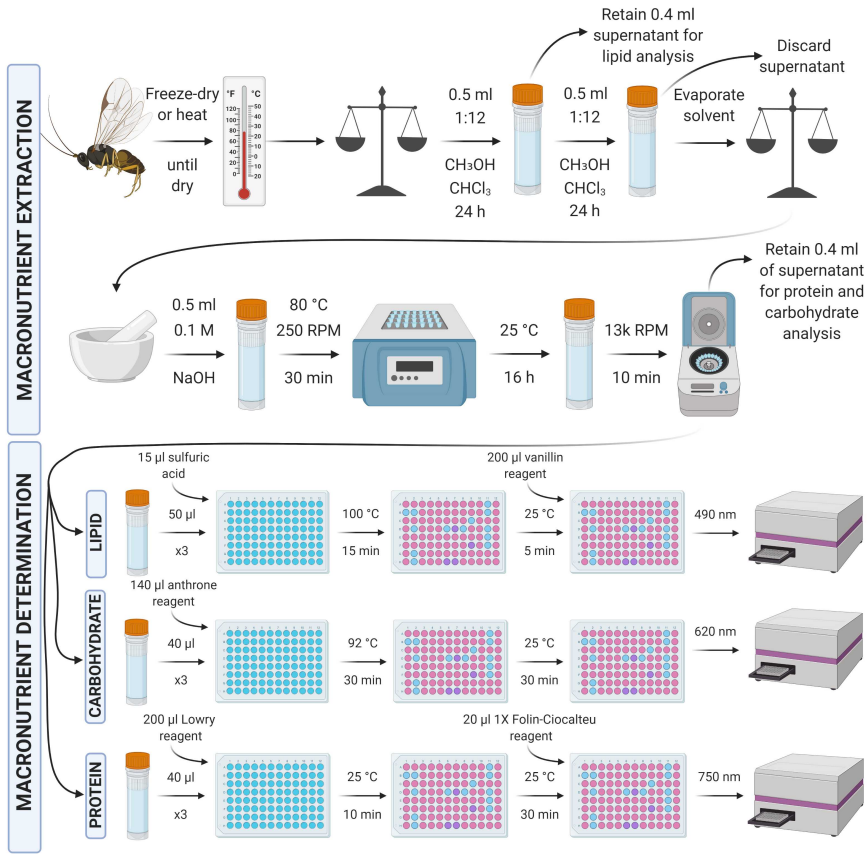
Welcome to MEDI!

1 Welcome to Macronutrient Extraction and Determination from Invertebrates (further micro-scaled edition)!

The following presents a micro-scaled version of the original MEDI protocol. The further micro-scaled protocol is intended particularly for invertebrates of dry volumes of 1 mg or less which cannot be pooled together given their scarcity or the focus of the study precluding pooling (e.g., investigation of individual differences). The micro-scaling is achieved by increasing analyte concentration at both the extraction and analysis stages, therefore facilitating more sensitive detection of macronutrients. Analytical standards must also be adjusted accordingly (detailed within).

Citation: Jordan Cuff (09/17/2021). Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates). https://dx.doi.org/10.17504/protocols.io.bw5hpg36

Here's an overview of the further micro-scaled extraction protocol:



An overview of the further micro-scaled MEDI protocol.

This is based on the protocol originally published in Methods in Ecology and Evolution:

> 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

Unlike the original MEDI protocol, this protocol does not include the determination of exoskeletal mass which may be an important factor in many studies. This could theoretically be incorporated into the protocol according to the instructions in the original protocol, but the miniscule masses that this further micro-scaled protocol is designed for would preclude the mass-based measurements except where access to highly specialised scales is possible.

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

Collection and preparation of materials 1d 4h 10m

2 Collect invertebrates and kill them, ideally by freezing or another optimally humane method. 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

Citation: Jordan Cuff (09/17/2021). Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates). https://dx.doi.org/10.17504/protocols.io.bw5hpg36

possible/appropriate/safe) to ensure all solubilised material is accessible for downstream analysis. This can be achieved during the drying described in step 3.

3 Desiccate the invertebrate specimens via freeze-drying (ideal) 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. Be wary of burning or otherwise damaging the bodies.

4 If possible, weigh the dried invertebrate using appropriately sensitive scales, recording the mass. 5m

This weighing is useful for representation of macronutrient contents as a proportion of total body mass, but the values can alternatively be presented as proportions in the absence of body mass (although this is sub-optimal).

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.

Determination of lipid content 1d 1h 35m

5 1d 0h 5m

Add 500 µl 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.

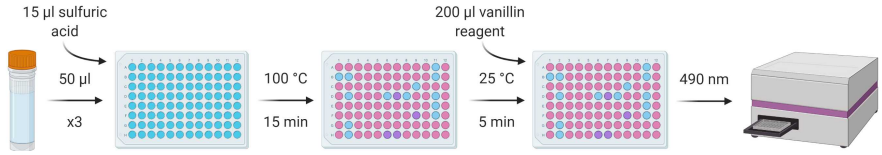
6 Remove 400 µl of chloroform:methanol solution and keep it in a separate tube for lipid analysis. The remaining solution in the original tube is taken forward to the next section (determination of protein and carbohydrate content; STEP 15), ideally with immediacy given that the next step is another overnight soak in chloroform:methanol. 10m

Ensure the tube containing the lipids is air tight to avoid evaporation, which will affect downstream concentration values. Consider placing the tubes in a freezer to further reduce evaporation. 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 (i.e., the paper is free of even residual lipids).

7 The lipid content of the 400 µl of chloroform:methanol solution removed during STEP 6 can be determined using the sulfo-phospho-vanillin method.

Citation: Jordan Cuff (09/17/2021). Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates). https://dx.doi.org/10.17504/protocols.io.bw5hpg36



8 Prepare a stock standard dilution series using a suitable analogue to the lipids present in your tissues (e.g. analytical lard oil for animals) of known concentration diluted with 1:12 chloroform:methanol. A dilution series of 0-1 mg/ml in nine increments (0, 12.5, 62.5, 125, 250, 375, 500, 750 and 1000 µg/ml) should suitably cover a broad range of concentrations, and can be adjusted further according to expected concentrations. 15m

9 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): 10m

A	B
Reagent	Amount
Vanillin	275 mg
Hot water	183 µl
85% phosphoric acid	46 µl

10 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). 15m

11 Add 15 µl concentrated sulfuric acid to the lipid residues and vortex/mix before incubating again at 100 °C for 15 min. 20m

This is one and a half times longer than in the original protocol. Leaving concentrated acids at high temperatures presents a number of possible hazards. Remain vigilant and keep close watch of the plate.

12 Allow the samples to cool to room temperature and add 200 µl vanillin reagent to each well, vortexing/mixing for homogenous colouration. 5m

13 After 5 min, transfer 200 µl from each well into a corresponding well in a new plate. 10m

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

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

Citation: Jordan Cuff (09/17/2021). Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates). https://dx.doi.org/10.17504/protocols.io.bw5hpg36

Determination of protein and carbohydrate content 1d 20h

15 1d 0h 10m

This continues with material generated in STEP 6.

From the original tube containing the remaining chloroform:methanol solution and the invertebrate tissue, discard the remaining ~100 µl supernatant (having already removed 400 µl for lipid analysis) avoiding the removal of any tissue. Add another 500 µl 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.

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

17 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 and any beads added are removed. 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.

18 Add 500 µl 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. 35m

19 16h

Allow the sample to soak overnight at room temperature.

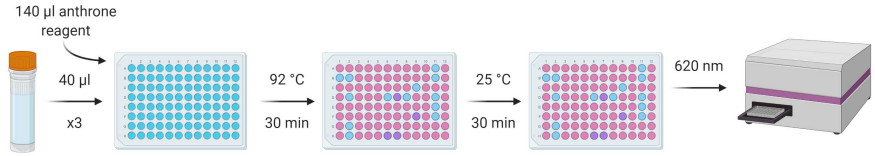
This overnight step can be left out if sure that the tissue is appropriately lysed, for example by including sonication and mixing halfway through.

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

21 Transfer 400 µl of the supernatant into a separate tube for taking forward to protein and carbohydrate determination. 5m

Citation: Jordan Cuff (09/17/2021). Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates). https://dx.doi.org/10.17504/protocols.io.bw5hpg36

22 The carbohydrate content of the 400 µl of NaOH solution removed during STEP 34 can be determined using the anthrone method.



23 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 0-0.02 mg/ml in nine increments (0, 0.25, 1.25, 2.5, 5, 7.5, 10, 15 and 20 µg/ml) should suitably cover a range of minute concentrations, but this can be adjusted accordingly. These concentrations were used for analyses of relatively carbohydrate-poor taxa like parasitoid wasps, thus more concentrated standards, e.g., a factor of ten more, may be better for other studies. Carbohydrate is typically much less concentrated in many invertebrate tissues compared to protein and lipid. 10m

24 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): 5m

A	B
Reagent	Amount
Anthrone	165 mg
Concentrated sulfuric acid	165 µl

25 From each standard and sample, put three repeats of 40 µl into a flat-bottomed 96-well plate, add 140 µl anthrone reagent to each and vortex/mix. 5m

26 Incubate the plate at 92 °C in a ventilated hood for 30 min. 30m

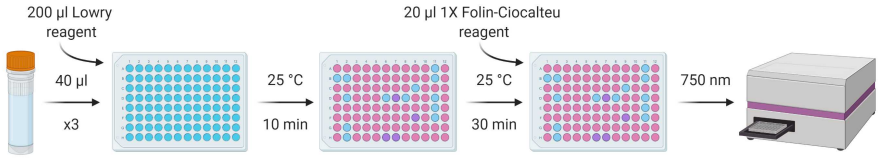
> This is three times longer than in the original protocol. Leaving concentrated acids at high temperatures presents a number of possible hazards. Remain vigilant and keep close watch of the plate.

27 Cool the plate to room temperature and incubate for a further 30 min to facilitate further development of the assay. 35m

28 Measure absorbance at 620 nm using a spectrophotometer. 5m

29 The protein content of the 400 µl of NaOH solution removed earlier 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 all viable, as are several other alternatives. This protocol will describe the protocol for the Lowry assay.

Citation: Jordan Cuff (09/17/2021). Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates). https://dx.doi.org/10.17504/protocols.io.bw5hpg36



30 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 0-1 mg/ml in nine increments (0, 12.5, 62.5, 125, 250, 375, 500, 750 and 1000 µg/ml) should suitably cover a broad range of concentrations, but this can be adjusted accordingly. 10m

Most protein assay kits will allow values to be converted for different standards using standard conversion factors since variation in the standards used will differentially affect the results of each assay.

31 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. 10m

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

33 Add 20 µl of 1X (1N) Folin-Ciocalteu reagent to each well and vortex/mix. 5m

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

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

Analysis of absorbance readings

36 You can now calculate the concentration of analyte based on the absorbance measurements. Keep in mind that the use of only 500 µl in each of the extraction phases means that mg/ml data will need dividing by two in order to calculate whole body macronutrient content. Any dilutions or other alterations to assay input concentrations/volumes must also be considered here.

Citation: Jordan Cuff (09/17/2021). Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates). https://dx.doi.org/10.17504/protocols.io.bw5hpg36