

VERSION 2
JAN 03, 2024

OPEN ACCESS



DOI:
dx.doi.org/10.17504/protocols.io.8epv5zp36v1b/v2

Protocol Citation: Jordan P Cuff 2024. Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates).
protocols.io
<https://dx.doi.org/10.17504/protocols.io.8epv5zp36v1b/v2>
Version created by Jordan P Cuff

MANUSCRIPT CITATION:
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>

Further Micro-scaled MEDI (Macronutrient Extraction and Determination from Invertebrates) V.2

Version 1 is forked from [MEDI: Macronutrient Extraction and Determination from Invertebrates](#)

Jordan P Cuff¹

¹Newcastle University

Foraging Ecology Research Group



Jordan P Cuff
Newcastle University

DISCLAIMER

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working
We use this protocol and it's working

Created: Jan 03, 2024

Last Modified: Jan 03, 2024

PROTOCOL integer ID:
92896

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

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.

IMAGE ATTRIBUTION

Figures created with Biorender.com.

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

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.

BEFORE START INSTRUCTIONS

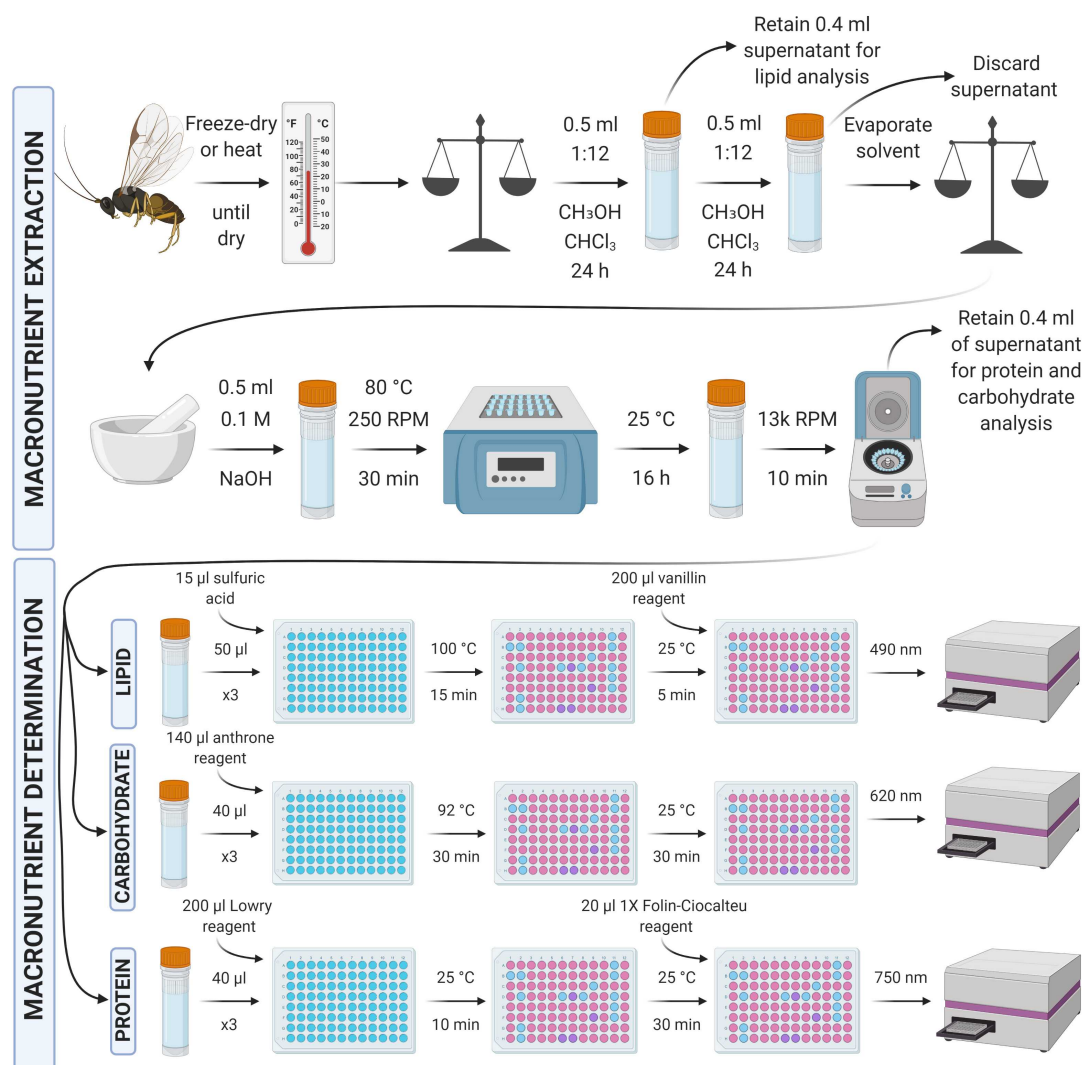
Please read the full protocol before starting and ensure that you have all the necessary equipment/reagents. Particular attention must be paid to the COSHH and safety documentation relating to the reagents. The original MEDI protocol is recommended for samples with dry masses greater than 1 mg. For samples 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).

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*:

CITATION

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*.

LINK

<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

Note

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/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

Note

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

Note

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).

Note

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** Add 500 μ l 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. **1d 0h 5m**



- 6** Remove 400 μ l 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**

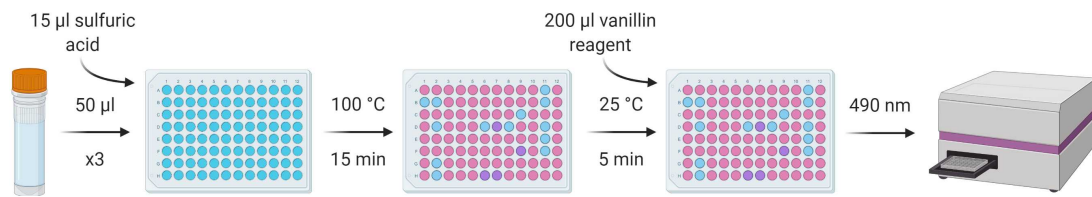
Note

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 μ l of chloroform:methanol solution removed during STEP 6 can be

determined using the sulfo-phospho-vanillin method.



- 8** Prepare a stock standard dilution series using a suitable analogue to the lipids present in your tissue (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

Note

High concentrations of chloroform (up to 100 %) are also viable, but be aware that this can interact negatively with some plasticware (especially during heated incubations), resulting in disruption to assay plates and pipette tips. Check compatibility first, ideally by heating a plate containing just the solvent.

- 9** Make up the vanillin reagent using the following amounts (which should account for reagent overage sample/standard repeat (so thrice per sample/standard if running triplicates, as advised): 10m

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

- 10** From each standard and sample, put three repeats of 50 µl into a flat-bottomed 96-well plate and heat 100 °C in a ventilated hood until all solvent has evaporated (leaving just the lipid residue; ~5-10 min). 15m
- 11** Add 15 µl concentrated sulfuric acid to the lipid residues and vortex/mix before incubating again at 100 °C. 20m

°C for 15 min.

Safety information

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

Note

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

Determination of protein and carbohydrate content

1d 20h

- 15 This continues with material generated in STEP 6. 1d 0h 10m



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.

Note

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

Note

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 thermo-shaker to ensure mixture of the lysed material with the NaOH. 35m

- 19 Allow the sample to soak overnight at room temperature. 16h



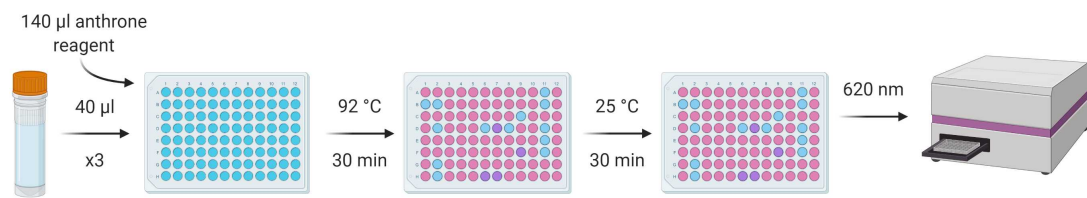
Note

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

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

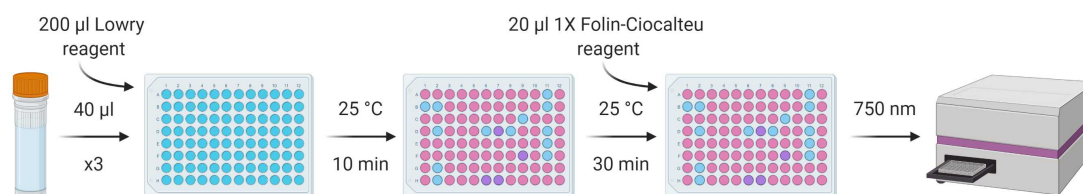
Safety information

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 μ l 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.



30 Prepare a stock standard dilution series using a suitable analogue (e.g. bovine serum albumin) of known concentration diluted with distilled water. A dilution series of 0-1 mg/ml in nine increments (0, 12.5, 62.5, 125, 250, 375, 500, 750 and 1000 μ g/ml) should suitably cover a broad range of concentrations, but this can be adjusted accordingly. 10m

Note

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 20 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.