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Biological applications of a new isothermal calorimeter that simultaneously measures at four temperatures

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Abstract Isothermal calorimetry is a powerful technique for the study of kinetics of physical, chemical, and biological processes, for example, of their temperature dependence. A new heat conduction calorimeter that simultaneously makes measurements on four samples at four different temperatures is presented in this article. Results from tests with four biological systems (milk fermentation, carrot juice spoilage, sunflower seed germination, and moss respiration) are shown. In all the cases, the instrument could measure the heat production rate—and thus the process rate—at the different temperatures used.

Keywords Isothermal heat conduction calorimeter · Biology · Temperature dependence · Arrhenius law · Milk fermentation · Carrot juice spoilage · Seed germination · Moss respiration

Introduction

Isothermal (heat conduction) calorimeters measure thermal power (heat production rate), P(W), which is proportional to the rate of the process being studied:

$$P = \Delta H \frac{\mathrm{d}n}{\mathrm{d}t} \quad (1)$$

In this article, ΔH (J mol^{-1}) is the reaction enthalpy, and dn/dt (mol s^{-1}) is the rate of the process. As nearly all processes (physical, chemical, and biological) produce heat, and as isothermal calorimetry directly gives a measure of the rate, isothermal calorimetry is an interesting method for studying kinetics of processes. This is especially true for the slow processes with time-scales from hours to years [1]. As measurements can be performed at different (constant) temperatures, it is also possible to evaluate activation energies (or other measures of the influence of temperature). However, a practical limitation for most laboratories is that such measurements are quite time consuming as the measurements have to be repeated at different temperatures. A study of a process that takes a week will, for example, take a month or more, if one needs to get data at 3–4 temperatures.

To improve the situation, the design of a novel isothermal (heat conduction) calorimeter that simultaneously measures the thermal power at four different temperatures is presented, and examples of its use in biological studies are provided.

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Instrument

The instrument consists of a tube of aluminum with a rectangular cross section (Fig. 1). The dimensions are 350 mm × 120 mm × 100 mm ($L \times H \times W$). At both ends of the tube, thermostated plates are placed. At the high-temperature end, the plate is heated by heater pads controlled by a PID regulator and a thermistor. At the low-temperature end, the plate is thermally connected to a thermoelectric cooling device (DA-075-24, SuperCool, Göteborg, Sweden) that is controlled by a PID regulator (PR-59, SuperCool, Göteborg, Sweden) and a thermistor. The temperature stability achieved under undisturbed

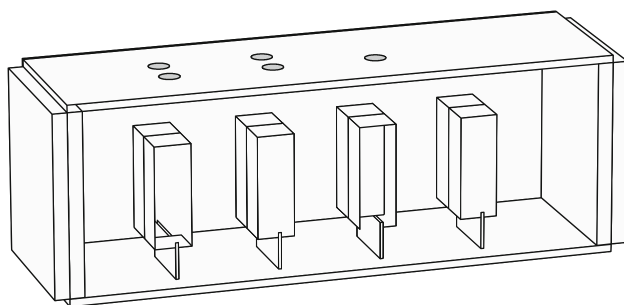

Fig. 1 Drawing of the calorimeter with the external insulation and one of the side plates removed. The four calorimeters are connected to the bottom plate by copper plates. The whole volume inside the tube—except the calorimeters and where the ampoules are inserted—is filled with insulation. On the left side, a cooling unit (not shown) is attached to cool the left side of the tube. On the right side, a heater controls the warm side temperature

ambient conditions is in the order of 1 and 10 mK at the heating and cooling ends, respectively.

As the plates at the end of the tube are set to different temperatures, a temperature gradient will be developed along the tube. To limit heat losses to the ambient and to protect the calorimeters from external disturbances, the whole device is placed in a 50-mm insulation of extruded polystyrene with a thermal conductivity of about 0.04 W m^{-1} K^{-1}.

Four small heat conduction calorimeters are placed at even distances inside the tube. They are connected to the bottom part of the tube by 2-mm copper plates that are placed at a right angle to the heat flow direction through the tube. In this way, each calorimeter is fixed at a single isothermal temperature. To prevent convection, the tube is filled with extruded polystyrene with a thermal conductivity of about 0.04 W m^{-1} K^{-1}.

Each calorimeter is a twin instrument with identical sample and reference calorimeters. The heat flow sensors (CP1.0-31-06L, Melcor, Trenton NJ, USA) are placed on a small aluminum heat sink. On the heat flow sensors are placed circular aluminum vial holders with an inner diameter of 14 mm. Tubes with square-shaped cross section and in contact with the heat sink are placed outside the heat flow sensors and the vial holders. The output voltages from the calorimeters are measured by an amplifier and data logger (ADC-24, Pico Technology, St. Neots, UK) on its most sensitive range (±39 mV, differential).

All the presented measurements have been made with 3-mL glass vials sealed with aluminum caps with rubber-PTFE septa or with silicon stoppers. The calorimeters were calibrated electrically using heaters in glass ampoules and electrical pulses of 500 s [2].

When a temperature gradient has been developed, the four calorimeters will be at different temperatures. At close to room temperature (when the heat losses to the ambient are low), there will be an approximately linear correlation between the distance along the tube and the temperature. The calorimeters are placed at even distances along the tube. Therefore, if the end plates have temperatures of, e.g., 10 and 35 °C, the calorimeters will be at 15, 20, 25, and 30 °C. It is thus easy to generate data to calculate the activation energy.

In the heat sink of each calorimeter is placed a Pt100 sensor (tolerance standard AA, i.e., ±0.1 K at 0 °C and ±0.2 K at 50 °C) connected to a Pt100 data logger (PT-104, PicoTech, St. Neots, UK).

The instrument is essentially a tube with a temperature gradient. The total cross-sectional area of the aluminum in the tube is 16.4 cm^2 and the length between the high and low temperature sides is 35 cm. If the heat losses through the insulation are disregarded, and a thermal conductivity of aluminum of 200 W m^{-1} K^{-1} is assumed, then the heat flow q (W) through the instrument is approximately

$$q = 1.0 \cdot \Delta T$$

where, ΔT (K) is the temperature difference between the warm and cold plates. This means that quite high powers are needed to sustain large temperature differences. For example, will a temperature difference of 40 K require 40 W of cooling on the cold side and the same thermal power on the warm side. This limits the temperature difference that is possible with an instrument of this type.

When the instrument is used with the cold side at temperatures less than the dew-point temperature, there will be condensation on the cold side. To avoid this, about 20 mL min^{-1} of dry air (room air that had passed through a column of dry molecular sieves) was pumped into the calorimeter's cold side.

Figure 2 gives the result of baseline measurements. As the temperature stability on the cold side was not as good as on the warm side (the thermoelectric cooling device is influenced by the room temperature), there was as a higher noise in the calorimeter with the lowest temperature, compared to the other three calorimeters. The stability of a baseline is almost in the microcalorimeter-range when the instrument is used in a room with constant temperature conditions.

A problem with the present instrument is that repeated baseline measurements often gave slightly different values. An example of this is given in Fig. 2 in which one of the channels shows a difference of 0.01 mW between two baseline determinations

Applications

We have used the calorimeter in studies on four different biological systems to test its potential as a producer of kinetic data.

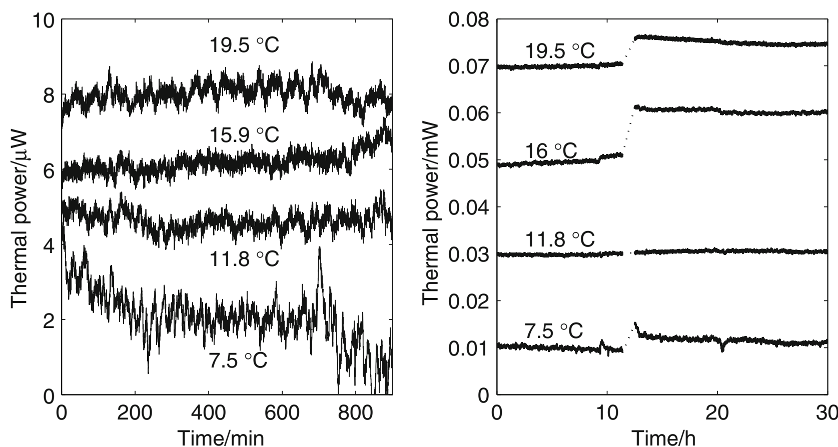

Fig. 2 Baselines measured at four temperatures (*left*) and the result of repeated charging of empty ampoules (*right*)

Milk fermentation

There are a large number of different fermented milk products on the marked in different countries. These are in most cases produced with lactic acid bacteria that both lower the pH and give the products a characteristic taste. We have made a study using the Swedish "filmjölk" type of fermented milk. This is a mixed culture of *Lactococcus lactis* and other bacteria that is fermented at around 20 °C.

A sample was prepared by mixing pasteurized milk (3% fat) with 1% of "filmjölk" as a starter culture. The four ampoules were then charged with (identical) 3-mL specimens taken from this sample. The measurements were made at four rather closely spaced temperatures: 23.6, 22.8, 21.8, and 20.8 °C.

The result of the milk fermentation measurements is seen in Fig. 3. An initial small peak possibly indicates the end of an initial aerobic phase before the main anaerobic phase produces the main heat. Integration of the measured heats (from 1.5 to 60 h) yielded 6.35 J g^{-1} at 23.6 °C, 6.89 J g^{-1} at 22.8 °C, 6.87 J g^{-1} at 21.8 °C, and 7.50 J g^{-1} at 20.8 °C. Possibly the higher result for the lowest temperature is the result of a less well-determined baseline at this temperature.

It is seen in Fig. 3 that the calorimetric curves follow in a logical order and that the instrument is capable of detecting differences in metabolic rates for even small temperature changes. Note that during the exponential growth phase are the thermal powers proportional to the bacterial growth rates and one can, e.g., calculate the doubling times for the exponential growth (1.71, 1.87, 2.06, and 2.26 h for the four temperatures in decreasing order of temperature).

Carrot juice spoilage

The shelf life of many foodstuffs is limited by microbial activity. This is true for such commodities as soft bread, cheese, and meat. Information about the rate of microbial growth is important for the prediction of shelf life as a function of temperature [3], which, together with water activity, is the most important factor for shelf life prediction for food stuff. A number of studies [4–7] have indicated that isothermal calorimetry is an interesting method for investigations of shelf life.

A sample of fresh carrot juice was prepared in a Bosch juice centrifuge and specimens of 3 mL of juice were charged into the calorimeter within 60 min of preparation. The measurements were run for almost 2 days at four temperatures ranging from a normal storage temperature to room temperature.

Figure 4 shows the result. It is seen that the microbiological degradation processes are clearly temperature dependent, and that the curves at different temperatures follow similar trends. It is also seen that the degradation processes are slow to start at 7.5 °C (the baseline is also more noisy at this temperature).

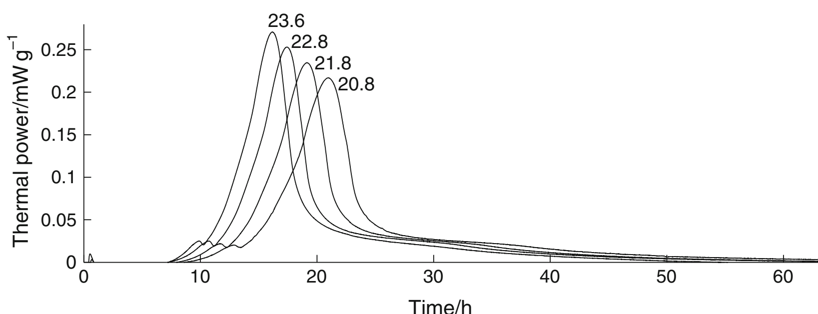

Fig. 3 Results from measurements on milk fermentation. The four curves give the results at 23.6, 22.8, 21.8, and 20.8 °C (from *left* to *right*)

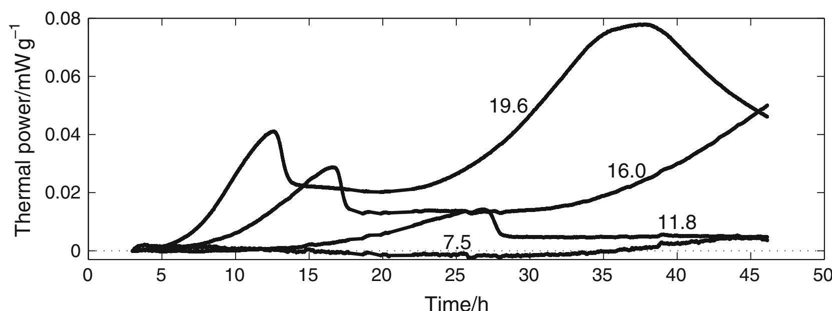

Fig. 4 Results from measurements on fresh carrot juice at four temperatures (given in the figure)

It is of interest to interpolate and possibly extrapolate results from shelf life measurements. One way to do this is to use the Arrhenius equation to model the temperature dependence of a rate constant or a rate of some sort. As an example of this, we have made an Arrhenius diagram, using the inverse of the time it took for the measured signals to exceed 1 μW g^{-1} as a rather arbitrary proxy for degradation rate. The result, shown in Fig. 5, gives a linear trend (constant activation energy) for the three lower temperatures. There is thus some rationale to use the Arrhenius equation to interpolate down to 7.5 °C (or even extrapolate to lower temperatures). The activation energy calculated for the three lowest temperatures is 120 kJ mol^{-1}.

Seed germination

Among the first to use isothermal calorimetry to study seed germination was Calvet and Prat [8] who, for example, investigated the temperature dependency of wheat germination. There have since then been a few calorimetric studies on seed germination presented, for example [9–11], and isothermal calorimetry is an interesting technique for following the germination of individual seeds or assemblages of seeds. A limitation is that—if one does not aerate the samples—the measurement time is limited by the oxygen content of the ampoule (and possibly also by the increase of the carbon dioxide concentration) [12]. In this article, a measurement on sunflower seeds in non-aerated vials is presented.

The measurement was made by dehulling sunflower seeds (*Heliantus annuus* var. Peredovik) and imbibing the seeds in water for 12 h. Three seeds were then placed on wet cotton in each vial. The measurements were made in closed vials and continued until the initially high thermal powers had dropped to near zero values because of the lack of oxygen (possibly in combination with increased levels of carbon dioxide).

Figure 6 shows the result of the seed germination measurements. The thermal powers are higher at higher temperatures, but oxygen depletion also comes earlier as the thermal power is a measure of the respiration rate (and the oxygen content is similar in the ampoules). The rates at the two highest temperatures are quite similar indicating that germination time is similar at 16 and 19.5 °C. This agrees qualitatively with published germination rates of sunflower seeds [13].

In Fig. 6 is also shown another way of evaluating the calorimetric results, which is by looking at the heat (the integral of the thermal power). One can then see that all curves end up at about the same level, indicating that the same factor (oxygen and/or carbon dioxide) have limited the germination process. The heat produced by the germination process comes from aerobic respiration, and the enthalpy for this process is 455 ± 15 kJ mol(O_2)$^{-1}$ [14]. Each vial contains about 2.2 mL air when it is closed and with the above enthalpy, this will generate about 8 J, which is in a fair agreement with the final heat seen in Fig. 6. The additional heat found in the measurement may be explained by that the water in the ampoule contained dissolved oxygen.

With aerobic biological systems, a closed ampoule experiment such as the one shown is useful as a control experiment. If differently sized samples of the same

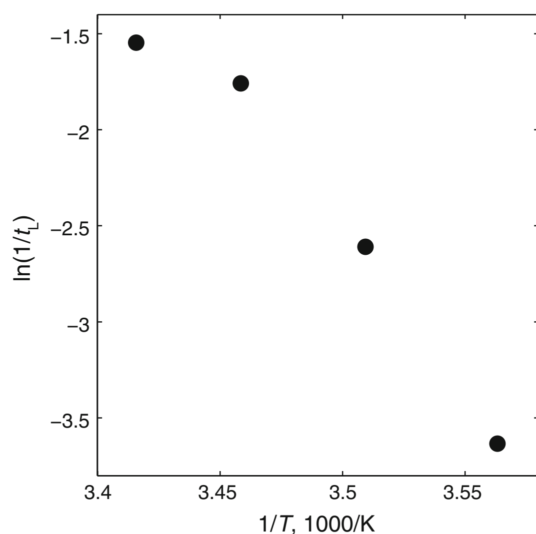

Fig. 5 An Arrhenius plot using the result in Fig. 4. The rate of the process at the four temperatures was taken as the inverse of the time t_L by which the exponential increase in thermal power reached 1 μW g^{-1}

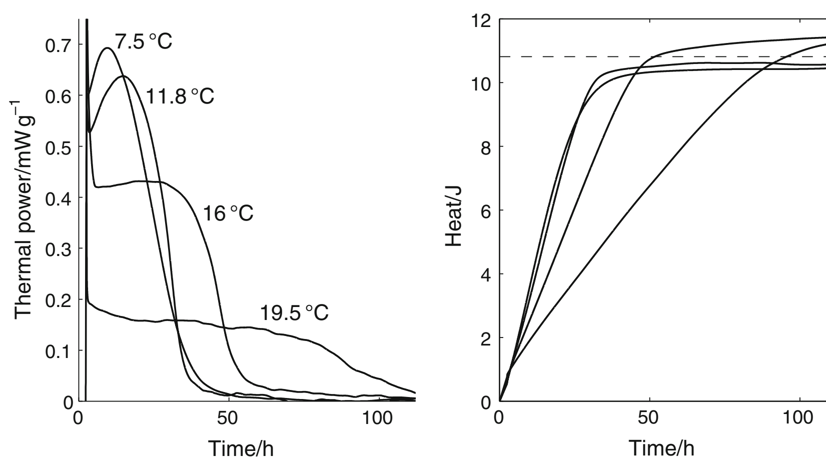

Fig. 6 Germination of sunflower seeds in closed ampoules at four temperatures. To the *left* is seen the primary result, and to the *right* is the integrated result. Note that the thermal power is in mW g^{-1}, but the heat is in J

aerobic tissue are measured until all oxygen is consumed, then the following should be observed:

- The total heat produced should be the same for all samples.
- The peak thermal power should be proportional to the size of the samples.
- The time until which a certain decrease in thermal power is seen should be inversely proportional to the size of the samples.

Note that some deviations from the above rules may be observed because of such factors as the presence of less air in an ampoule with more sample, and water likely containing dissolved oxygen.

Moss activity

Mosses belong to the kingdom of plants in a division called Bryophytes or non-vascular plants. They can be found worldwide from the arctic regions to the tropics, but chiefly in moist habitats with low light intensities. As they are plants, they contain chlorophyll and therefore do photosynthesis, but in contrast to “real” plants, they have no root system and instead absorb water directly through the whole leaf area. Some species can survive desiccation for long time periods, and their photosynthesis and metabolism will be fully restored again only a few hours after being wetted again [15–17].

There is a long history of measurements by isothermal calorimetry for different purposes on plants and plant tissue (e.g., [8]). Calorimetry has also been shown to be a useful tool for the study of, e.g., metabolic kinetics as a function of temperature [18–23]. It should be noted that although the photosynthesis is necessary for plants' survival, studies of the efficiency of the photosynthesis is not the best way to measure growth parameters. This is because it is not the photosynthesis, but the rate and efficiency of building biomass from the products of photosynthesis, which determine the actual growth rate of an organism [24]. Calorimetric heat rate measurements under dark conditions can thus give information on the temperature dependence of the growth rate of an organism.

The measurement on mosses was different from, for example, the milk fermentation measurement in that it was not possible to make four identical samples, and it is also not possible to use the initial or final part of a measurement as a baseline. Because of this, the authors instead measured each sample at all four temperatures and used baselines from a separate baseline determinations with vials with water (with similar heat capacity as the samples) before all measurements. The measurement with each sample was ended at the same temperature as the one at the start of the measurement, so that it could be seen if the respiration rate had decreased during the experiment. The measurement program is given in Table 1. As mosses—in contrast to other samples used in this study—are photoautotroph organisms, the moss samples had to be exposed to light between the measurements as will be described later.

The sample material of the moss (*Tortula ruralis* (Hedw.) Gärtn., Meyer&Scherb.) used in this study was collected from an east-facing 45° roof in the southern-most part of Sweden. The roofing material was asbestos fiber cement slates. The moss sample was precultivated in a chamber with a 16 h + 8 h light–dark cycle (PPFD = 20–40 µmol m^{-2} s^{-1}) at 10 °C. Before the measurements, the bottom quarter of each moss branch was cut off together with the substrate to which it was attached (sand, asbestos fibers, organic matter etc.). The remaining top parts were charged into four ampoules. Each ampoule was filled with about 0.24 g of moss branches, still leaving some headspace to avoid anaerobic conditions during the measurements. Tap water (0.48–0.6 mL) was then added to each ampoule with a syringe, making sure that all parts of the samples were humidified. This gave the moss samples

Table 1 The measurement schedule used for the moss measurements

	T1	T2	T3	T4
bl + $h\nu$1	–	–	–	–
mea 1	S1	S2	S3	S4
bl + $h\nu$2	–	–	–	–
mea 2	S2	S3	S4	S1
bl + $h\nu$3	–	–	–	–
mea 3	S3	S4	S1	S2
bl + $h\nu$4	–	–	–	–
mea 4	S4	S1	S2	S3
bl + $h\nu$5	–	–	–	–
mea 5	S1	S2	S3	S4
bl 6	–	–	–	–

First a baseline was measured at the same time as the moss samples were exposed to light (bl + $h\nu$1). Then the samples were measured (mea 1). This procedure was repeated four more times with the samples (S1–S4) being shifted to a new temperature (T1–T4) each time, so that the samples were at the same temperatures at the first and last measurement. Finally a baseline measurement was made. In the evaluation the initial baseline was used

moisture contents equal to or above 200%. The open ampoules were then placed in a humidity box at 100% RH in the 10 °C chamber for 3 days. Before the first calorimetric measurement, the samples were kept under light for 10 h (PPFD = 20–40 μmol m^{-2} s^{-1}).

The calorimetric measurements of the four moss samples were carried out according to Table 1. The sealed ampoules were measured on in the calorimeter for 2 h, opened and placed in the humidity box under light for 2 h, sealed, and measured on for 2 h. The samples were weighted after each measurement to verify whether there had been any moisture loss (the mass loss was never more than 0.006 g). After the series of five measurements, the moss ampoules were dried in an oven at 105 °C during 24 h to get the dry weight.

The result of the calorimetric measurements of the moss samples can be seen in Fig. 7. All the samples show similar results, and, as expected, each sample produces less heat at lower temperatures. There are also relatively small differences between the first (1) and the last (5) measurements for each sample, which were made at the same temperatures, indicating that the samples were not damaged by the measurement program.

Discussion

The measurements presented above show that the instrument presented is capable of isothermally measuring thermal powers at four different temperatures at the same time. The baselines noise is in the order of 1 microwatt, but repeated baseline measurements often gave different values (Fig. 2). One explanation for the low baseline repeatability is that the output of the calorimeters is sensitive to the ampoule positions. In contrast to ordinary isothermal calorimeters that are placed inside thermostatic baths, these calorimeters are placed in a temperature gradient. Even if the calorimeters are arranged so that each calorimeter should be fixed at a certain temperature, there are temperature gradients over the calorimeters that are much

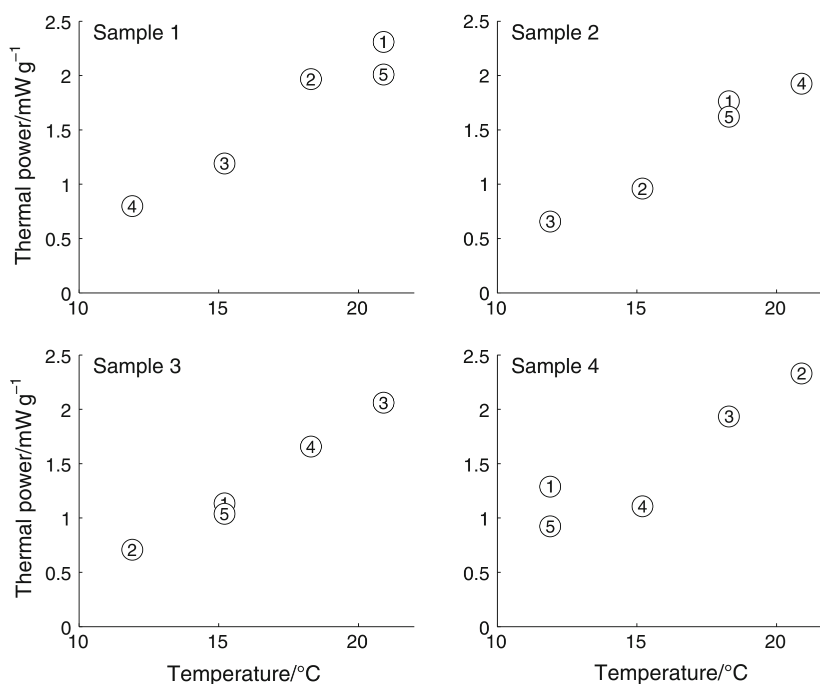

Fig. 7 The result from the measurements on four moss samples. Each sample was measured five times as described in Table 1 (the number of each measurement is given in the figures)

higher than those found in ordinary heat flow calorimeters. Small differences in, e.g., how an ampoule is placed (for example on which side most of the air–gap between ampoule and ampoule holder is placed), may give significant differences in the heat flow patterns, and also in the results. This may be a limitation with this kind of instrument, but the situation may possibly be overcome with novel ampoule arrangements that fix the ampoules in the same position each time.

In the measurements conducted in this study, the uncertainties in the baseline determinations were problematic for the moss measurements as it was not possible to take a baseline during these measurements. In this case, a separately measured baseline was used, and this increased the uncertainty of the measurement as the baselines were not stable, as discussed above. This was not a problem in, e.g., the milk fermentation measurements as one can evaluate a baseline in the beginning of such an experiment, before the growth is detected.

The instrument considered here can generate kinetic data as a function of time for four temperatures. If the rate, but not the process path, is temperature dependent, then calorimetry is a powerful method to generate data for modeling kinetics [1, 25]. Although biological systems clearly are so complex that they do not fully satisfy the above criteria, the output from isothermal calorimetry (thermal power) is still an interesting parameter to use in modeling and prediction studies. In the first two examples in this article (milk fermentation and carrot juice spoilage), the measured curves had similar shapes, but had lower peak thermal powers and were more extended in time at lower temperatures. The results are clearly useful for different types of interpolations and extrapolations, e.g., "at what temperature is 95% of the fermentation finished at 40 °C?" and "When does the thermal power from this spoilage processes exceed a threshold of 10 μW g^{-1} at 5 °C?".

In the latter two examples in this article (seed germination and moss activity), the aim of the measurements is to quantify the activity of a biological system as a function of temperature. In the case of the germinating seeds, the result is influenced by the oxygen depletion in the closed ampoules. This did not happen in the moss measurements case, as the thermal powers (respiration rates) were much lower for the moss samples than for the seed samples. The latter was also measured during much shorter time. However, in both cases, it was possible to evaluate the thermal power as a function of temperature.

Conclusions

A newly developed isothermal calorimeter that measures at four temperatures at the same time has been presented in this study. It was tested and found to work well in a number of biological applications. Its main limitation is that there are sometimes comparatively high baseline shifts when a sample is recharged, something that may be an inherent feature of this type of instrument.

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