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Thermochimica Acta 251 (1995) 119–130

thermochimica acta

Energetic investigation of *Saccharomyces cerevisiae* during transitions. Part 1. Mass balances ☆

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Received 28 July 1994; accepted 2 August 1994

Abstract

The calculation of material balances during the transitions implies the evaluation of cumulated quantities consumed and produced. This article presents the techniques to achieve this goal by evaluating the quantities involved in the reactions at each observation and by calculating the material balances (carbon, nitrogen, degrees of reduction) at each observation. Balance profiles are assessed by statistical tests and an experiment involving different metabolic pathways is analyzed.

Keywords: Mass balance; Metabolism; *Saccharomyces cerevisiae*; Transition

List of symbols

F	flow rate ($l\ s^{-1}$)
h	test function
i	corresponds to the ith species
in	input

☆ Presented at the Ninth Conference of the International Society of Biological Calorimetry, Berlin-Schmerwitz, 27–31 May 1994.

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SSDI 0040-6031(94)02043-4

j	corresponds to the jth observation
K	number of constraints
k	corresponds to the kth balance
n	number of moles (mol)
$\mathbf{n}$	vector of moles (mol)
out	output
S	species concentration (mol l^{-1})
s	number of species
t	time
V	volume (l)
$\mathbf{X}$	element fraction matrix
$x_{k/i}$	molar fraction of element k in species i
$1-\theta$	significance level of χ^2 distribution
χ^2	chi-square probability distribution
ε	error on balance (mol)
$\boldsymbol{\varepsilon}$	vector of the errors on balances (mol)
γ	degree of reduction
Σ	variance–covariance matrix of the balance
Ψ	variance–covariance matrix of the data (mol^2)

1. Introduction

Growth processes can involve different substrates and products. Hence it is necessary to check mass and energy balances in order to calculate yields and to quantify metabolism. Each atomic species or intensive quantity must be conserved: the most common independent balances concern carbon, nitrogen, enthalpy and degrees of reduction. All forms of these entities must be measured, therefore balances for oxygen or hydrogen atoms cannot be directly calculated because water is produced. Techniques to investigate balances were developed for continuous cultures [1–3] in which the environment of the microorganisms is stable. This paper presents a method for checking the balances during transitions, i.e. when the reactor is not at steady state, for example after a shift in the dilution rate or during a batch or a fed-batch experiment.

2. Definition of the system

Fig. 1 shows the boundary of the open system: substrate enters and broth leaves the fermentor. There is heat exchange with the environment.

The input feed rate is F^{in} (l s^{-1}) at concentration S^{in} (mol l^{-1}). We consider s different species and consider S_i for one of them ($i = 1-s$). The molar output flow rate is $S_i \cdot F^{out}$ (mol s^{-1}). The heat output Q, expressed in W, is directly measured with a calorimeter.

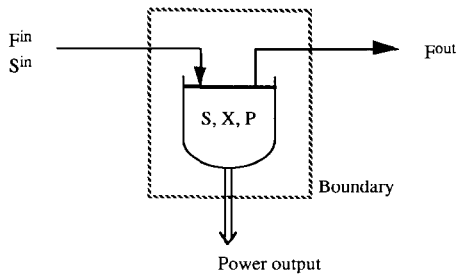

Fig. 1. Boundary of the system.

3. Mass balance

A mass balance for any species S_i gives across the boundary [4]

$$\frac{dn_i(t)}{dt} = \frac{d[S_i(t)V(t)]}{dt} - S_i^{in}(t)F^{in}(t) + S_i(t)F^{out}(t) \quad (1)$$

where n_i is the number of moles consumed or produced by the reaction and the left hand term of the equation is the net reaction rate of the species S_i. The first term on the right is the accumulation rate, the second the input feed rate, and the last the output feed rate.

All the variables are independent of time at steady state, and there is no accumulation because the content of the reactor does not change with time.

4. Accumulated quantities

The accumulation term between the $(j-1)$th and the jth observation is not negligible during transitions because there is no steady state. The mass balance equation (1) can be integrated between two observations at times t_{j-1} and t_j

$$n_i(t_{j-1} \to t_j) = [S_i(t)\ V(t)]_{t_{j-1}}^{t_j} - \int_{t_{j-1}}^{t_j} S_i^{in}(t)F^{in}(t)\ dt + \int_{t_{j-1}}^{t_j} S_i^{out}(t)F^{out}(t)\ dt \quad (2)$$

Each term is estimated by the method of trapezoids (Fig. 2): for example, the quantity that has left the reactor between t_{j-1} and t_j is approximated by the shadowed area of the trapezium

$$\int_{t_{j-1}}^{t_j} S_i(t)F^{out}(t)\ dt \approx \frac{S_i(t_{j-1})F^{out}(t_{j-1}) + S_i(t_j)F^{out}(t_j)}{2}(t_j - t_{j-1}) \quad (3)$$

The same technique is applied to all the terms to estimate the net increment of each species S_i for each time interval t_{j-1} to t_j. Let us call $n_i(j)$ the number of moles involved in the reaction since the beginning of the experiment: by definition,

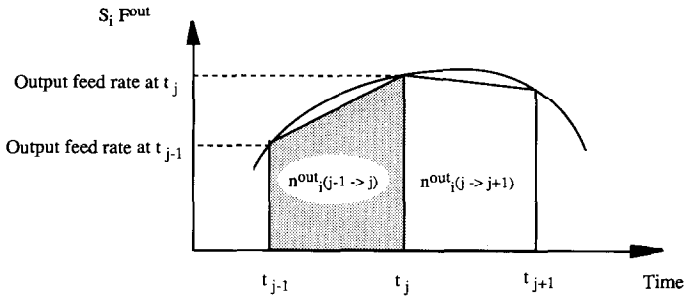

Fig. 2. Integration by the method of trapezoids.

no reaction has been observed before the first sample at time t_1. Cumulated quantity $n_i(j)$ involved between the observations at time t_j and t_1 (beginning of the experiment) is easily calculated by the sum of each increment

$$n_i(j) = n_i(t_1 \rightarrow t_2) + n_i(t_2 \rightarrow t_3) + \ldots + n_i(t_{j-2} \rightarrow t_{j-1}) + n_i(t_{j-1} \rightarrow t_j) \quad (4)$$

and $n_i(j)$ can be expressed in moles, grams, optical density units etc.

We define $\mathbf{n}(j)$ as the vector containing the number of mole produced or consumed up to the jth observation.

The graph of $\mathbf{n}$ vs. time represents the forward profile of the cumulated quantities. The quantity increases if the species is produced and, conversely, decreases when the species is consumed. The quantity is constant when the net production rate is zero, i.e. when the species is neither consumed nor produced, or when the consumption and production rates are equal.

5. Linear constraints

5.1. Error measurement

It is necessary to check the consistency of data before performing any calculation. In fact, it is difficult to describe the metabolism accurately or to quantify biochemical pathways if some relevant species is omitted or biased. Nevertheless, some scatter in the data has to be accepted because of uncertainties in the analytical methods and acceptable errors. We consider K balances [1] concerning C, N and the degrees of reduction. We define k, one of these elements ($k = 1-K$).

For each element e_k, the net quantity of element consumed in the reactor must be equal to the net quantity of element produced. Let the symbol n_i equal the cumulated quantity of species i and $x_{k/i}$ the element molar fraction of k in i. We construct the matrix $\mathbf{X}$ of element fraction of which each element X_{ki} is equal to $x_{k/i}$.

For s different species containing the element k, the balance on that element is

$$\varepsilon_k = \sum_{i=1}^{i=s} n_i x_{k/i} \quad (5)$$

The constraint is that the balance should equal zero. It is possible to investigate the balances individually (each balance should equal zero) or simultaneously. In that case, the combined balance is a vector of which each element is one of the balances, and the constraint is that each element of the vector should equal zero. The vector of balances is denoted as $\boldsymbol{\varepsilon}$ and the general formula using the matrix notation is

$$\boldsymbol{\varepsilon} = X\,\mathbf{n}^{\mathrm{T}} \tag{6}$$

where the superscript T denotes the transposition operator.

The next section will focus on the material balances for an experiment involving glucose, ethanol, biomass, CO_2, O_2 and ammonium ions. The second part of this series (Part 2. Energy balance and thermodynamic efficiency) will concern the enthalpy balance.

5.2. Carbon balance

The elemental composition of the biomass is assumed to be constant. The composition [5] per mode of C: $CH_{1.65}O_{0.53}N_{0.15}$ and the ash content is 8.34 wt%. The ash-free molecular weight is $MW_{\text{ash-free}} = 24.27$ (g mol^{-1} of C). The molecular weight of the measured biomass is $MW_X = 26.48$ (g mol^{-1} of C). The cumulated biomass is expressed in moles of C of biomass.

The molar carbon fractions are $x_{C/\text{glucose}} = 6$, $x_{C/\text{ethanol}} = 2$, $x_{C/CO_2} = 1$, $x_{C/\text{biomass}} = 1$, and the carbon balance is $\varepsilon_c = 6n_{\text{glucose}} + 2n_{\text{ethanol}} + n_{\text{biomass}} + n_{CO_2}$.

5.3. Nitrogen balance

The source of nitrogen is ammonium, and the only product containing nitrogen is biomass. The balance involves only two species and the results depend mainly on the accuracy of the determination of the nitrogen content of the biomass ($x_{N/\text{biomass}} = 0.15$)

$$\varepsilon_N = n_{NH_4^+} + x_{N/\text{biomass}} n_{\text{biomass}}$$

5.4. Degree of reduction balance

The degree of reduction of a compound S_i of elemental formula $C_{x_{C/i}}H_{x_{H/i}}O_{x_{O/i}}N_{x_{N/i}}$ is defined per mole of C

$$\gamma_i = \frac{4x_{C/i} + x_{H/i} - 2x_{O/i} - 3x_{N/i}}{x_{C/i}} \tag{7}$$

The values for our example are

$$\gamma_{\text{glucose}} = 4,\ \gamma_{\text{ethanol}} = 6,\ \gamma_{\text{oxygen}} = -4,\ \gamma_{\text{biomass}} = 4.13$$

and the degree of reduction balance is

$$\varepsilon_\gamma = 6n_{\text{glucose}}\gamma_{\text{glucose}} + 2n_{\text{ethanol}}\gamma_{\text{ethanol}} + n_{\text{biomass}}\gamma_{\text{biomass}} + n_{O_2}\gamma_{O_2}$$

5.5. Time profile

The balances are calculated for each observation j with the quantities $n_i(j)$. We obtain a time profile of each balance calculated between the first and the jth observation. The evolving analysis of the balances is a powerful tool to detect new events or changes during the experiment. Our general goal remains to satisfy the balances at the end of the experiment, i.e. for the last observation.

5.6. Statistical test

Each material balance on cumulated quantities should be verified at each observation. However, even if there is no gross error of measurement, the balance will not be exactly equal to zero owing to the random noise in the measurements. The most simple test for checking if ε_k is acceptable is to compare ε_k with the input. This gives the fraction of the feed that is missing or in excess. The value can be compared with the estimated accuracy of the measurements and gives a first criterion for evaluating a balance. Nevertheless, this method does not provide an absolute threshold value to reject or accept the balance. Statistics are more suitable to detect errors.

Several authors [2,3] have proposed a statistical test to investigate the balances once we have accepted a certain level of noise in the experimental data. We assume that the experimental data are normally distributed with a known variance–covariance matrix Ψ. Under these assumptions, the balances $\boldsymbol{\varepsilon}$ are normally distributed and the variance–covariance of $\boldsymbol{\varepsilon}$ is Σ

$$\Sigma = X^{\mathrm{T}} \Psi X \tag{8}$$

We want to test whether the balance is close enough to zero so that we can attribute the residual distance from zero to the random noise. For that purpose, we calculate the weighted distance to the target (here, the distance from zero). The weight is the inverse of the variance–covariance matrix Σ of $\boldsymbol{\varepsilon}$. Now we calculate the statistical function h

$$h = \boldsymbol{\varepsilon}^{\mathrm{T}} \Sigma^{-1} \boldsymbol{\varepsilon} \tag{9}$$

This function is distributed as a central χ^2 distribution with K degrees of freedom if the hypothesis that the balances are equal to zero is correct [3]. The test function h calculated with K balances is also called the Mahalanobis distance, because it is the weighted distance to the target zero, and it is compared with a χ^2 distribution with K degrees of freedom and a level of significance $1-\theta$. This gives us an upper control limit (UCL). If $h > \chi^2(1-\theta, K)$ the hypothesis concerning the balances is rejected; conversely if $h < \chi^2(1-\theta, K)$ the hypothesis cannot be rejected. For a level of significance of 95%, the UCL is 2.71 for one degree of freedom, 4.61 for two degrees of freedom, 6.25 for three degrees and 7.78 for four degrees of freedom. The appendix gives a numerical example of the calculation.

Plotting h versus time indicates subsets of data for which the hypothesis might be rejected. The first observations usually do not satisfy the statistical test because even low levels of noise have a large effect on the h value. A significant result necessitates at least 10 observations.

6. Materials and methods

Saccharomyces cerevisiae CBS 426 was grown aerobically in continuous cultures in an RC1 bench scale calorimeter (Mettler Toledo, Greifensee, Switzerland) with a working volume of 1.5 l. The heat signal was monitored on-line. The medium contained, per liter: 20 g glucose, 9 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.6 g $MgSO_4$, 0.3 g $CaCl_2$, 0.7 g NaCl, 0.1 ml antifoam (polypropylene glycol P2000) and 1 g yeast extract (Oxoid). The pH was adjusted to 5 by the addition of 4 N NaOH. The temperature set point was 30°C and the stirring rate was 600 rpm. The aeration rate was 0.85 l min^{-1} (0.6 vvm). The partial oxygen pressure was always above 50% of saturation.

The outlet gas was heated at 45°C to avoid condensation of water and ethanol. The concentration of oxygen was determined with a paramagnetic analyzer (Servomex, Crowborough, UK). Carbon dioxide and ethanol in the off-gases were monitored by infra red analyzers (Servomex, Crowborough, UK).

Glucose, ethanol, acetic acid, glycerol and ammonia were determined enzymatically (Boehringer), and phosphate and protein were determined spectrophotometrically.

Biomass concentration was determined by filtration of a 6 ml sample on 0.45 μm membranes. Membranes were pre-weighed and then dried for 48 h at 100°C.

7. Results and discussion

The culture was grown aerobically at 0.1 h^{-1} and the dilution rate was shifted to 0.40 h^{-1}, far above the critical dilution rate. Production of ethanol and acetic acid immediately set in. The biomass concentration decreased owing to product formation and to a transient imbalance between the growth rate and the dilution rate (which increased by a factor of 4). Six hours after the shift-up and before the new steady state was reached, the dilution rate was decreased to 0.05 h^{-1}. There was no nitrogen consumption for 2 h. Ethanol was no longer produced and consumption was evident 2 h after the shift-down. Acetate accumulated in the broth and was rapidly consumed after exhaustion of the ethanol. The biomass concentration increased.

This experiment involved the oxidation of glucose, ethanol and acetate and the reduction of glucose to ethanol.

The investigation of the material balances is the topic of the next section. The energy balance is the topic of Part 2 of the series (Energy balance and thermodynamic efficiency).

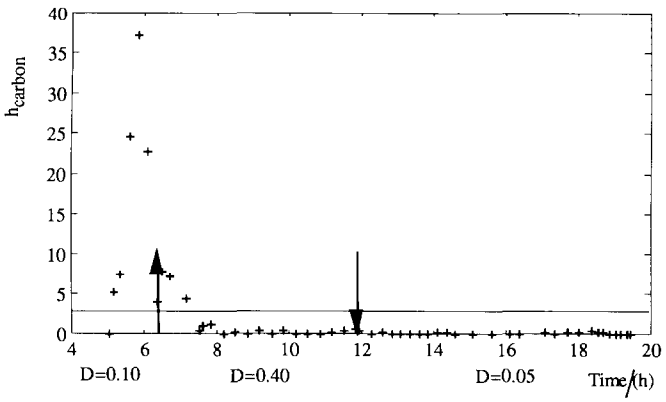

Fig. 3. Test value of carbon balance profile. The arrows indicate the shift-up and the shift-down respectively. The horizontal line shows the upper control limit for rejecting the hypothesis.

7.1. Carbon balance

It is necessary to monitor all the compounds containing carbon: glucose, ethanol in the liquid and the gaseous phase, acetate, glycerol, CO_2 and biomass. In our case the ethanol accumulated in the gaseous phase is 6.65% of the accumulated ethanol in the liquid phase. Total organic carbon analysis shows that the residual carbon is derived from residual glucose, ethanol, acetate and yeast extract. We consider that yeast extract is not a major carbon source, but it may be a source of nitrogen. A simplified numerical example is given in the appendix.

Cumulated quantities and carbon balance have been calculated for each sampling time. Fig. 3 shows the profile of the test function for the carbon balance. At the end of the experiment there is a deficit of 0.42% compared with the carbon fed to the reactor. The χ^2 test shows that the hypothesis is never rejected (Fig. 3), except for some observations at the beginning of the experiment, where the calculation is very sensitive to errors. At the end of the experiment the test value is $h = 0.015$ compared with an UCL value of 2.71 for the distribution of χ^2 with one degree of freedom and $\theta = 0.1$. There is absolutely no reason to reject the carbon balance.

7.2. Nitrogen balance

Ammonium is the source of nitrogen; yeast extract may also be a source of N but it is neglected. Biomass is the only product containing nitrogen, so that the balance involves only two species: ammonium and biomass.

The balance represents 3.94% of the nitrogen fed to the reactor at the end of the experiment. The error is more important at a dilution rate of 0.40 h^{-1} ($\approx$20%) and the statistical test is failed (Fig. 4). Errors may be due to a changing biomass composition. In fact, nitrogen content is related to enzyme content, and it has been shown that nitrogen content increases with increasing dilution rates. The amount of enzyme necessary to metabolize higher substrate fluxes is higher. In addition,

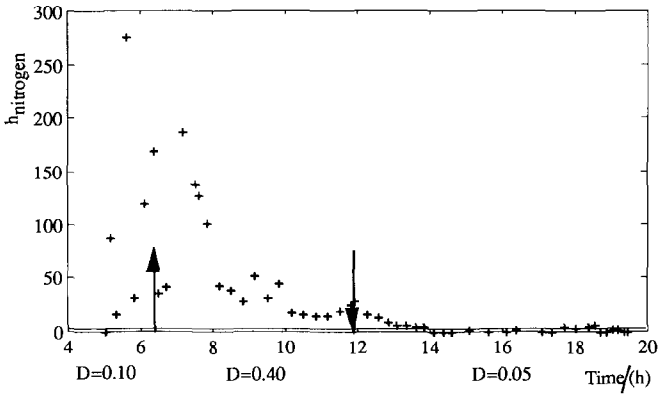

Fig. 4. Test value of nitrogen balance profile. The arrows indicate the shift-up and the shift-down respectively. The horizontal line shows the upper control limit for rejecting the hypothesis.

another source of error may be the yeast extract. Nevertheless, we cannot reject the nitrogen balance since the final test value h_N is only 0.039, compared with the Upper Control Limit of 2.71.

7.3. Degree of reduction balance

The species involved are: glucose ($\gamma = 4$), ethanol ($\gamma = 6$), acetate ($\gamma = 4$), biomass ($\gamma = 4.13$), and oxygen ($\gamma = -4$). The degrees of reduction of CO_2 and of NH_4^+ are zero. At the end of the experiment the balance represents 2.37% compared with the incoming degrees of reduction (glucose and oxygen) and the final test value is $h = 0.306$, which is much lower than the UCL of 2.71. The degree of reduction balance is also accepted. Fig. 5 represents the statistical test function versus time,

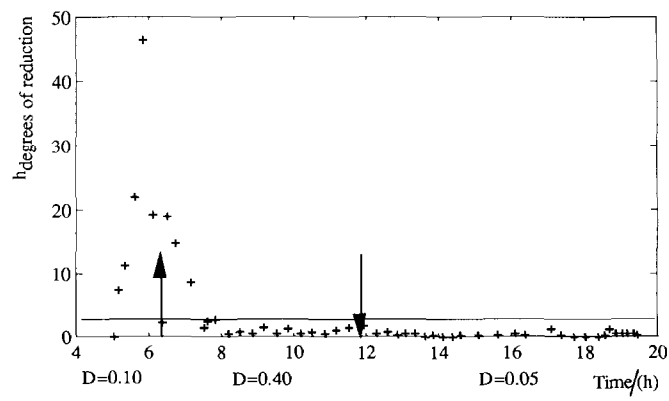

Fig. 5. Test values of degrees of reduction balance profile. The arrows indicate the shift-up and the shift-down respectively. The horizontal line shows the upper control limit for rejecting the hypothesis.

and shows that the hypothesis is rejected only for some points at the beginning of the experiment.

7.4. Combined statistical test

We can test the hypothesis that the three balances are satisfied simultaneously, which is quite different from investigating the three balances independently. Let us consider a case where the three balances are accepted but each test function is close to the Upper Control Limit. We assume for each balance that chance (i.e. the noise on the measurements) can explain the gap relative to perfect balances. Nevertheless, if we investigate the three balances simultaneously the situation is different. Chance is not longer sufficient to justify three simultaneous gaps, whereas single balances are accepted. We are more demanding when we perform a combined statistical test by investigating several balances together.

The χ^2 distribution has three degrees of freedom because three balances are implied and the threshold value is 6.25. At the end of the experiment the test value was 3.506. We cannot reject the hypothesis that the balances concerning carbon, nitrogen and the degrees of reduction are simultaneously correct.

7.5. Balance profiles

The analysis of Figs. 3–5 is useful for detecting changes in the reactions. The χ^2 test can be rejected mainly for the following reasons: errors in measurement, a major compound is not measured, the estimated variance on species is too low, the biomass composition is not correct.

This last hypothesis is usually neglected, but several authors have shown that the biomass composition depends on the strain, the growth condition and the dilution rate. Fig. 4 shows that the nitrogen balance is rejected until the shift-down. The nitrogen fraction of biomass is assumed to be constant. The value was determined by Larsson et al. [5] at 0.11 h^{-1}. This value seems not to be acceptable for 0.40 h^{-1}, but neither is it acceptable to use the nitrogen content at 0.4 h^{-1} because this value was determined for a culture at steady state. This illustrates one of the limits of balances: the need for accurate parameters gained under the same working conditions, which is sometimes not possible in a changing environment.

7.6. Indirect balancing

If the molar fraction of one element is unknown, it might be possible to estimate this value if the balance were correct. For example, the carbon fraction of biomass varies little with dilution rate, whereas nitrogen and phosphorus vary: nitrogen is involved in protein and phosphorus in RNA. The biomass content of these two elements increases with increasing dilution rates as the specific substrate flux is more important. The content is estimated by calculating the ratio on cumulated

quantities: for example, for nitrogen and biomass at the j-th observation

$$x_{N/biomass}(j) = \frac{n_N(j)}{n_{biomass}(j)} \tag{10}$$

This yield is calculated for each observation. The amounts of species consumed and biomass produced should vary enough to be influenced by changes. One can see that they are inflenced by the dilution rate:

at $D = 0.40\ h^{-1}$, $x_{N/biomass} = 0.18$ N-mol/C-mol and

$x_{P/biomass} = 0.023$ P-mol/C-mol

at $D = 0.05\ h^{-1}$, $x_{N/biomass} = 0.15$ N-mol/C-mol and

$x_{P/biomass} = 0.017$ P-mol/C-mol

8. Conclusions

Transient metabolism can differ largely from what is observed at the steady state. Material balances are important to check the consistency of data. They can be calculated on flows at steady state or on quantities accumulated during transitions to take into account the term of dilution.

The article shows an application with different products and substrates. Statistical testing can facilitate the interpretation of the balances, and the rejection of the balances can be due to errors in the analysis, neglect of a major species consumed or produced, the assumed elemental properties of biomass not being constant during the experiment.

Acknowledgments

Financial support from the Swiss National Science Foundation (FNRS) is gratefully acknowledged. We thank David Jemielity for correcting the manuscript.

Appendix

In our example of calculation of the test function, we consider the last observation and the carbon balance. We will neglect the ethanol in the vapor in order to simplify the notations of this example.

The vector of the species is

$$\mathbf{n} = [\text{glucose} \quad \text{ethanol} \quad \text{biomass} \quad CO_2 \quad \text{acetate}]$$

The experimental values at the end of the experiment are (in moles)

$$\mathbf{n} = [-0.461 \quad 0.331 \quad 1.055 \quad 0.991 \quad 0.014]$$

The carbon constraint is simply the molar carbon content of each species

$$X = [6 \quad 2 \quad 1 \quad 1 \quad 2]$$

and the carbon balance is

$$\varepsilon = (-0.461) \times 6 + 0.331 \times 2 + 1.055 + 0.991 + 0.014 \times 2 = -0.0116 \text{ mol of C}$$

The distance from zero is: $\varepsilon^2 = (0.0116 - 0) \times (0.0116 - 0) = 0.000134$.

We assume that all the variables are measured with 3% error: the variance–covariance matrix Ψ of the measurement is a diagonal matrix with the elements $\Psi_{1,1} = (-0.461 \times 0.03)^2$, $\Psi_{2,2} = (0.331 \times 0.03)^2$, $\Psi_{3,3} = (1.055 \times 0.03)^2$ etc...

The variance–covariance matrix of the balance is a scalar equal to 0.0092. The test function is calculated with Eq. (9). The numerical value is

$$h = \frac{0.000134}{0.0092} = 0.0146$$

We compare this value with a χ^2 distribution with one degree of freedom and a level of significance of 90%. h is far below the upper control limit of 2.71. This means that the distance from zero is not large enough to reject the hypothesis that it is equal to zero. The level of noise that we have accepted in the data (3%) is sufficient to explain the difference of the simplified carbon balance from zero.

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