New Method to Determine the Mass Transfer Resistance of Sterile Closures for Shaken Bioreactors

Tibor Anderlei, Christian Mrotzek, Stefan Bartsch, Ghassem Amoabediny, Cyril P. Peter, Jochen Büchs

Biochemical Engineering, RWTH Aachen University, Worringenweg 1, 52056 Aachen, Germany; telephone: +49-241-8025546; fax: +49-241-8022256; e-mail: buechs@biovt.rwth-aachen.de

Received 23 November 2006; revision received 31 March 2007; accepted 26 April 2007
Published online 11 May 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21490

ABSTRACT: In this paper a novel and easily applied method to measure the mass transfer resistance of the sterile closures (e.g. cotton plug) of shaken bioreactors is introduced. This method requires no investment in special equipment (e.g. an oxygen sensor) and can be performed with the materials usually available in typical laboratories. The method is based on the model of Henzler et al. (1986), which mechanistically describes mass transfer through the sterile closure of a shaken bioreactor based on diffusion coupled with Stefan convection. The concentration dependency of the multi-component diffusion coefficients is taken into account. The water loss from two equivalent shaken bioreactors equipped with sterile closures during several days of shaking is measured. One flask contains distilled water, the other a saturated salt solution. From the water evaporation rate in each of the two flasks, the new model presented calculates the relative humidity in the environment, the average diffusion coefficient of oxygen in the sterile closure ($D_O2$), and the diffusion coefficient of carbon dioxide ($D_CO2$). The diffusion coefficient of carbon dioxide ($D_CO2$) only depends on the density and material properties of the sterile closure and not on the gas concentrations and is, therefore, an ideal parameter for the characterization of the mass transfer resistance. This new method is validated experimentally by comparing the diffusion coefficient of oxygen ($D_O2$) to a measurement by the classic dynamic method; and by comparing the calculated relative humidity in the environment to a humidity sensor measurement.


KEYWORDS: aeration; mass transfer resistance; oxygen transfer rate; sterile closure; shake flask; water evaporation; relative humidity

Introduction

In early stages of the biotechnological process development, shaken bioreactors are still the most widely used bioreactors. Their low investment cost and ease of handling allow for a large number of fermentation experiments to be conducted in parallel. Cultivation of aerobic microorganisms, however, requires a sufficient oxygen supply to the culture media, because a limitation of oxygen could affect bacterial growth and product formation (Finn, 1954; Steel, 1958; Yamada et al., 1978). Impeded mass transfer from the culture medium also influences the removal rate of volatile compounds like CO2, ethanol, inducers, etc. and therefore has to be adjusted in a suitable range. Consequently, a crucial parameter for the design of suitable operating conditions is a gaseous compound’s maximal possible transfer rate from the environment to the culture medium and is termed as the mass transfer capacity.

The mass transfer capacity of the shaken bioreactor is determined by two resistances, namely the gas–liquid interface and the sterile closure (e.g. a cotton plug). The gas–liquid mass transfer has been described in numerous publications (Corman et al., 1957; Drew, 1981; Elmer and Gaden, 1962; Flach and Heden, 1963; Freedman, 1969; Humphrey, 1998; Maier and Büchs, 2001; Maier et al., 2004; McDaniel and Bailey, 1969; Solomons, 1969) and is not in the focus of this study. The mass transfer resistance of sterile closures however is investigated by three different methods: First, the classic dynamic method (Chain and Gualandi, 1954; Hirose et al. 1966; Schultz, 1964; van Suijdam et al., 1978; Yamada et al., 1978) employs an oxygen probe in the headspace of the shake flask. This head space is flushed with nitrogen until the oxygen concentration reaches zero. At this point, the flask is exposed to ambient air and the nitrogen...
supply is stopped. From the initial increase of the oxygen partial pressure, a simple mass transfer resistance of the sterile closure is calculated. Second, a steady state method was reported by Henzler et al. (1986). During fermentation with two parallel shake flasks containing the same microbial culture, one of the flasks was sealed in order to stop the mass transfer with the environment. The sustained respiration activity of the culture led to a change of the gas composition in the headspace of the shake flask. Samples from the gas phase of the flask were analyzed in a mass spectrometer, from which the authors calculated the gas–liquid mass transfer rate of oxygen and carbon dioxide. A comparison with the composition of the gas phase of the unsealed shake flask allowed evaluation of the mass transfer resistance of the sterile closure. The mathematical description not only considered diffusion but also a counter flowing convection, the so-called Stefan convection. Finally, Mrotzek et al. (2001) presented a method by coupling the model of Henzler et al. (1986) with the water evaporation rate from the shake flask. For this purpose, water vapor was considered as a fourth relevant gas component, in addition to oxygen, carbon dioxide and nitrogen. The water evaporation rate, that is the mass transfer of water vapor across the sterile closure was measured and then correlated to the mass transfer of oxygen and carbon dioxide. In this method, however, the relative humidity in the environment, which may be subject to climatic changes during the measuring period, must be monitored by a humidity sensor.

All three methods described above require expensive specific equipment (e.g. an oxygen probe, a mass spectrometer, a humidity sensor) or time-consuming manpower (e.g. for sampling) preventing their use in everyday laboratory work. In contrast, this work aims at developing a new method that can be extremely easily applied to determine the mass transfer resistance for all relevant gaseous compounds across various sterile closures. A procedure will be described which only requires a standard balance most probably available in every laboratory.

Theory

Mass Transfer Through a Sterile Closure

Henzler et al. (1986) have described the mass transfer through a sterile closure under steady state conditions: In contrast to older literature (Chain and Gualandi, 1954; Hirose et al., 1966; Schultz, 1964; van Suijdam et al., 1978; Yamada et al., 1978) which only consider Fick’s diffusion, Henzler et al. (1986) have shown that convection also plays an important role in the mass transfer through the closure. Convection occurs because of non-equimolar gas consumption and production by microorganisms, when the respiration coefficient RQ is not equal to one, and because of water vapor transfer. Figure 1 shows a schematic drawing of a shake flask closed with a cotton plug, illustrating the gas exchange across the sterile closure. The following equation describes the total volumetric flow \( Q_i \) of a gas component \( i \) across the sterile closure:

\[
Q_i = -D_i A \frac{d(p_i/p)}{dh} + wA \frac{p_i}{p} \tag{1}
\]

The first term of the equation expresses the diffusion. It should be noted that the diffusion coefficient \( D_i \) in a multicomponent system depends on the gas composition and therefore is a local value. The second term represents the convective flow (Stefan flow) characterized by the velocity \( w \). Defining the height coordinate \( h \), Mrotzek et al. (2001) have shown experimentally that the size of the sterile closure outside the flask neck has no significant influence on the mass transfer resistance. Therefore, only the flask neck is taken into account and the height coordinate \( h \) may originate at the upper edge of the flask. To access the velocity \( w \), steady state conditions are considered, and thus the total flow of the inert gas component nitrogen \( i = N_2 \) can be set to zero:

\[
Q_{N_2} = 0 \tag{2}
\]

For this case, Equation (1) may be rearranged for the velocity \( w \):

\[
w = D_{N_2} \frac{d(p_{N_2}/p)}{dh} \frac{p}{p_{N_2}} \tag{3}
\]

Inserting Equation (3) into the generalized equation (1) then leads to an expression for the cross-area specific volumetric flow rate of the component \( i \) across the sterile closure:

\[
q_i = \frac{Q_i}{A} = -D_i \frac{d(p_i/p)}{dh} + D_{N_2} \frac{d(p_{N_2}/p)}{dh} \frac{p_i}{p_{N_2}} \tag{4}
\]
Furthermore, Equation (4) contains the local diffusion coefficient \(D_i\), which changes with the height of the closure due to gas composition profile. Wilke (1950) have described this dependency by:

\[
D_i = \frac{1 - p_i/p}{\sum_{j=2}^{n} \frac{p_j}{p}}
\]  
(5)

This equation relates the diffusion coefficient \(D_i\) of the component \(i\) in a multicomponent mixture with \(n\) components to the binary diffusion coefficients \(D_{ij}\), which do not depend on the gas composition. For the diffusion coefficient of carbon dioxide \(D_{CO_2}\), Equation (5) may be written as follows:

\[
D_{CO_2} = \frac{1 - p_{CO_2}/p}{\frac{p_{O_2}/p}{D_{CO_2/O_2}} + \frac{p_{N_2}/p}{D_{CO_2/N_2}} + \frac{p_{H_2}O/p}{D_{CO_2/H_2}O}}
\]  
(6)

To simplify this equation, the following assumptions are made: As the gases nitrogen and oxygen have very similar kinetic properties (Perry and Green, 1994), the difference in the diffusion coefficients \(D_{CO_2/O_2}\) and \(D_{CO_2/N_2}\) are negligible. Moreover, the partial pressure of water vapor \(p_{H_2}O\) is 25-fold lower than the partial pressure of nitrogen and can therefore be neglected, while the diffusion coefficient \(D_{CO_2/H_2}O\) is in the same order of magnitude as \(D_{CO_2/N_2}\) (Henzler et al., 1986). Finally, the total pressure \(p\) of a multicomponent gas mixture is defined as the sum of the partial pressures:

\[
p = \sum p_i = p_{O_2} + p_{CO_2} + p_{N_2} + p_{H_2}O
\]  
(7)

In conclusion, these assumptions lead to

\[
D_{CO_2} \approx D_{CO_2/N_2}
\]  
(8)

and therefore, the diffusion coefficient of carbon dioxide \(D_{CO_2}\) is independent of the gas composition. Consequently, the diffusion coefficient of carbon dioxide \(D_{CO_2}\) depends only on the material properties of the sterile closure (e.g. density of cotton in a plug) and therefore is, according to Henzler et al. (1986) and Mrotzek et al. (2001), an ideal measure to characterize the mass transfer resistance of sterile closures.

Mrotzek et al. (2001) have used Equations (1)–(8) to calculate the mass transfer through a number of different sterile closures, based on the evaporation rate of water through these closures. This approach is supported by the works of Lockhart and Squires (1963) who already found a significant correlation between the aeration and the evaporation of water in a shaking flask. Other authors (Aiba et al., 1973; Sumino and Akiyama, 1987) reported the close relationship of the transfer coefficients between oxygen and water vapor. Figure 2 shows the structure of the model. The method is based on the measurement of the water evaporation rate \((ER)\) from a shake flask, closed with the sterile closure to be investigated. This evaporation rate is used in turn as an input variable for the model, which calculates the diffusion coefficient of carbon dioxide in the sterile closure. Moreover, the following parameters are needed: The average relative humidity outside the shake flask \(RH_{\text{out}}\), the temperature \((T)\), and the geometry factor \((F)\) of the shake flask neck

\[
F = \frac{A}{H}
\]  
(9)

according to Figure 1. As an initial value for the iteration, a more or less arbitrary value of \(D_{CO_2,0}\) has to be provided. The model then calculates the relative humidity in the headspace of the shake flask:

\[
RH_{\text{in,calc}} = \frac{\rho_{H_2}O|H=H}{p}
\]  
(10)

In the case of relevant deviations between the calculated value \(RH_{\text{in,calc}}\) and the real relative humidity within the headspace \((RH_{\text{in}} = 100\%)\), the iteration value of \(D_{CO_2}\) (see above) is adapted and used as a new input value for the next iteration step. This optimization procedure is repeated until the accuracy \(\epsilon\) is reached, resulting in the diffusion coefficient of carbon dioxide \(D_{CO_2}\) which ultimately characterizes the mass transfer resistance of that sterile closure.

In contrast to the constant diffusion coefficient of carbon dioxide \(D_{CO_2}\), the diffusion coefficient of oxygen \(D_{O_2}\) is dependent on the local gas composition in the sterile closure (cf. Eq. 5). In order to obtain a parameter for practical use, however, the calculated local values across the height of the sterile closure are averaged:

\[
\overline{D_{O_2}} = \frac{\int_0^H D_{O_2} dh}{H}
\]  
(11)

---

**Figure 2.** Model structure of Mrotzek et al. (2001) for the determination of the diffusion coefficient of carbon dioxide \(D_{CO_2}\). Measured input variables: water evaporation rate and outside relative humidity.
This method requires the relative humidity outside of the shake flask as average value during the measuring period ($RH_{out}$) as a known parameter and, therefore, it has to be measured using dedicated equipment.

**Elimination of the Average Relative Humidity Measurement**

To eliminate the relative humidity of the environment ($RH_{out}$) measurement, a new approach employing an extended experiment and a modified model calculation is developed in the following: Instead of one shake flask as in the method by Mrotzek et al. (2001) described above, two shake flasks are employed in the new, extended method. One shake flask is filled with deionized water, resulting in a relative humidity in the headspace of the shake flask of $RH_{in} = 100\%$. Additionally, a second flask containing a saturated salt solution is used. A saturated salt solution leads to a decreased and defined relative humidity in the corresponding gas phase above (Greenspan, 1977). If both shake flasks are closed with the same sterile closure to be investigated, and are operated on the same shaker in the same room, they both have the same temperature and the same relative humidity in the environment ($RH_{out}$). Therefore, the driving concentration difference of water vapor across the sterile closure in each flask is different, and two different evaporation rates will be measured, which are in turn fed into the extended model. It should be noted that a changing value for $RH_{out}$ during the measuring period does not interfere with the applicability of the proposed method.

The structure of the new extended model is depicted in Figure 3, of which one part, namely for the deionized water flask, is exactly the same as the model of Mrotzek et al. (2001) described above (Figure 2). The second part, however, has the same structure, but is parameterized for the shake flask containing the saturated salt solution with a lower relative humidity in the head space. These new parameters are marked with an asterisk ($\ast$). While the measured evaporation rates (ER, $ER\ast$), the relative humidities in the flasks ($RH_{in}, RH_{in}\ast$), and the geometry factors ($F, F\ast$) are specific for each of the two flasks, the relative humidity ($RH_{sink}$), the temperature ($T$) and diffusion coefficient of carbon dioxide ($D_{CO2}$) are common variables. In conclusion, each of the model parts calculates the respective relative humidity ($RH_{in,calc}, RH_{in,calc}\ast$) with a separate respective ER, ER\ast, but with a common $D_{CO2}$. Under the assumption of the mass transfer of the sterile closure being the rate determining step, this common $D_{CO2}$ is in turn characteristic for the sterile closure investigated. As long as the deviation between the calculated relative humidities ($RH_{in,calc}, RH_{in,calc}\ast$) and the real relative humidities ($RH_{in}, RH_{in}\ast$) in the headspaces of the shake flasks is higher than the defined tolerance ($\varepsilon$), the diffusion coefficient of carbon dioxide ($D_{CO2}$) and average relative humidity ($RH_{out}$) are optimized. As a result, this method returns calculated values for the relative outside humidity.

**Figure 3.** Extended model structure for the determination of the outside relative humidity ($RH_{out}$) and the diffusion coefficient of carbon dioxide ($D_{CO2}$) with two shake flasks operated in parallel, one filled with deionized water and the other one with a saturated salt solution under the same operating conditions.
without requiring to measure it and for the diffusion coefficient of carbon dioxide as a value characterizing the mass transfer resistance of the sterile closure.

Materials and Methods

Shake Flasks and Operating Conditions

All experiments were performed with Erlenmeyer flasks (DIN 12380 for narrow necked and DIN 12385 for wide necked geometry) with nominal flask volumes as indicated for each experiment. The flasks were mounted on a rotary shaker (LS-W, Adolf Kuehner AG, Basel, Switzerland). The standard operating conditions for all experiments were set to very commonly applied values: A shaking diameter of 50 mm, a shaking frequency of 200 rpm and a constant temperature of 24°C.

The sterile closures investigated were ordinary, handmade cotton plugs, and industrially produced paper plugs (Steri-Stopfen, VWR International, Darmstadt, Germany).

Measurement of the Water Absorption of the Sterile Closure

During the experiment for the measurement of the water evaporation rate from the shake flask through the sterile closure to be investigated, some water is adsorbed by the sterile closure itself. To access this amount, wide necked and narrow necked shake flasks with a nominal volume of 250 mL were filled with 10% of the nominal flask volume of deionized water (25 mL) and shaken under the standard operating conditions. The sterile closure was weighted before and several times during the experiment. While weighing the closure, the flask was closed with a lid to prevent any water loss to the environment. This experiment was conducted in triplicates for each neck type.

Measurement of the Relative Humidity Inside the Flask

Two hundred and fifty millilitres narrow and wide necked shake flasks were modified by addition of two connections in the head space of the flask: One valve for gas addition which will be needed for the dynamic method explained further below and one fitting for a humidity sensor (MiniCapII, Panametrics GmbH, Hofheim, Germany). The shake flasks were filled with 10% of the nominal flask volume of deionized water and shaken under the standard operating conditions.

To measure the relative humidity in the environment (RH_{in}) of the shake flasks, a second humidity sensor of the same type as specified above was mounted in the surrounding air. This measure is needed as an input variable for the calculation described by Mrotzek et al. (2001).

Measurement of the Water Evaporation Rate

To measure the water evaporation rate from which the mass transfer resistance is calculated by the model described further above, shake flasks with nominal volumes of 50, 100, 250, and 500 mL were employed. For each experiment, one shake flask was filled with 10% of the nominal flask volume of deionized water. A second flask with the same nominal volume and the same filling volume was filled with a saturated, aqueous sodium chloride solution, resulting in a relative humidity in the flask headspace of RH_{in} = 75.3% (Greenspan, 1977). Some experiments were also conducted using KOH (RH_{in} = 33.07%) and MgCl\(_2\) (RH_{in} = 9.32%). Both flasks were closed with the same type of sterile closure. Each shake flask including the closure was weighted after an equilibration phase which will be investigated separately, to obtain the initial weight m_0. The measuring time (t) was varied between 140 and 320 h. After the measuring time, the shake flask (including the closure) was weighted again to obtain the final weight m_1. From these two values, the water evaporation rate (ER) was determined by:

\[
ER = \frac{m_1 - m_0}{t} \tag{12}
\]

Dynamic Method for the Measurement of the Mass Transfer Resistance

The dynamic method was employed in order to test the method of Mrotzek et al. (2001). The 250 mL narrow necked flask to measure the relative humidity described further above is closed with an ordinary, handmade cotton plug and shaken at standard operating conditions. An oxygen sensor (250 G, Ceramatec, Salt Lake City, UT) was introduced to the flask headspace to measure the oxygen partial pressure (p_{O_2,in}). The headspace of the flask was flushed with nitrogen via the extra valve and thereby oxygen was removed from the gas and liquid phase. When the oxygen partial pressure (p_{O_2,in}) in the headspace of the flask (V_G) was nearly zero, the nitrogen supply was stopped. The oxygen partial pressure in the flask headspace (p_{O_2,in}) was then recorded over time. From the increase rate, the average oxygen diffusion coefficient (\(D_{O_2}\)) could be calculated after Aiba et al. (1973):

\[
D_{O_2} = - \frac{HV_G}{A} \ln \left( \frac{p_{O_2,in}}{p_{O_2,in}^0} \right) \tag{13}
\]

Experiments employing smoke as a tracer have shown that the gas phase may be regarded as well mixed, agitated by the rotating liquid during shaking.

Software

All models were implemented in Modelmaker (V3.0.3, Cherwell Scientific Publishing Ltd., Oxford, UK) and Matlab (V5.2.0.3084, MathWorks, Natick, MA).
Results and Discussion

The new method for the evaluation of the mass transfer resistance of sterile closures consists of several steps, as described in the theoretical part. To investigate its applicability, it was tested in several aspects. First, the assumptions were investigated, namely the amount of water adsorbed by the sterile closure and the water saturation of the flask head space itself. Second, the general model structure was tested on the deionized water part only. For this purpose, the average diffusion coefficient of oxygen as calculated from the water evaporation rate was compared to the results from the classic dynamic method. Finally, the new method was validated in whole by comparing the relative humidity in the environment (RH<sub>out</sub>) as calculated by the model with the measured value. Some results on the mass transfer resistance of typical sterile closures for shake flasks are presented.

Water Absorption of the Sterile Closure and Relative Humidity in the Flask Headspace

Figure 4 shows the weight increase of a cotton sterile closure, relative to the weight of a dry closure at the beginning of the evaporation experiment. It can be seen that in all flasks, the relative weight of the sterile closure increased by about 0.75–1.25% during the first 8 h. After this time, the relative weight remained practically constant, indicating a saturation of the closure with water after 8 h.

To test the water saturation of the flask headspace, wide and narrow necked flasks of nominal volumes of 100, 250, 500, 1,000, 2,000, and 5,000 mL filled with deionized water, and closed with ordinary, handmade cotton plugs were shaken for 24 h. After this time, the humidity RH<sub>in</sub> was found 100% for all flasks investigated (results not shown). Therefore, this time span is considered sufficient to saturate the flask headspace with water vapor, even for a flask with the largest nominal volume of 5,000 mL. In conclusion, an equilibration phase of a 24 h was set, before the experiment to determine the water evaporation rate from the shake flask was started in order to ensure steady state conditions during a water evaporation experiment.

Validation of the General Model Structure

To validate the general model structure described further above, the evaporation rate from different shake flasks filled with deionized water was measured. These measured evaporation rates were used as input variables for the model Mrotzek et al. (2001) (Fig. 2), which provides not only the diffusion coefficient for carbon dioxide, but also the average diffusion coefficient of oxygen (D<sub>O2</sub>). This calculated average oxygen diffusion coefficient was then compared to a value measured with the dynamic method as expressed in Equation (13). Figure 5 shows the average diffusion coefficients of oxygen (D<sub>O2</sub>) determined by both methods for 250 mL shake flasks with narrow and wide neck geometry, both equipped with ordinary, handmade cotton plugs. The respective values of the average diffusion coefficient of oxygen (D<sub>O2</sub>) determined by the two methods show remarkably small deviations of lower than 5%.

In order to substantiate the reproducibility of the new method, a series of experiments was carried out, first measuring the water evaporation rate and then calculating the diffusion coefficient of carbon dioxide of the sterile closure by the model of Mrotzek et al. (2001). Figure 6 shows the calculated diffusion coefficients of carbon dioxide from six experiments with 250 mL narrow necked shake flasks, closed with an industrially produced paper plug, and for six experiments with 250 mL wide necked shake flasks, closed with an ordinary, handmade cotton plug. It can be seen that the deviations between the average carbon dioxide diffusion...
coefficient of the narrow necked shake flasks are lower (<2%) than the deviations between the values of the wide necked flasks (<7%). The lower deviation of the narrow necked flasks is attributed to the industrially produced paper plugs, which are very stable and uniform in structure. In contrast, the closures of the wide necked flasks were handmade cotton plugs, which bear higher deviations in physical structure and properties. The mass transfer characteristics of each closure type will be discussed in detail further below. Taken together, the method of Mrotzek et al. (2001), which is also employed in the new method described in this work, is well suitable for the determination of the diffusion coefficient of carbon dioxide ($D_{CO2}$) and the characterization of the mass transfer resistance of the sterile closure.

Validation of the New Method

The new method substitutes the direct measurement of the relative humidity of the environment (RH$_{out}$) and eliminates the humidity sensor from the experimental procedure. To test this new method, the water evaporation rate was measured in two identical flasks, equipped with the same sterile closure, one filled with deionized water, and the other with a saturated sodium chloride solution. These evaporation rates were used as input variables into the new model (Fig. 3), which calculates the diffusion coefficient of carbon dioxide and the relative humidity in the environment. As a reference, the relative humidity in the environment was also measured by a humidity sensor. Four different narrow necked shake flask sizes with a nominal flask volume of 50, 100, 250, and 500 mL were investigated. Figure 7 shows the average relative humidity in the environment for five independent experiments, given by the climate during the specific time period of the respective experiments. The results are calculated by the new model and measured by the humidity sensor. It can be seen that there is a remarkable agreement between the calculated and the measured values. The average deviation of the two methods is lower than 2.2%. These results show that the relative humidity in the environment (RH$_{out}$) can be calculated by the new extended model very accurately. At the same time, the diffusion coefficient of carbon dioxide may serve as a characteristic value for a specific sterile closure. It is therefore an easy-to-apply method not requiring any expensive equipment.

Mass Transfer Resistance of Different Sterile Closures

Figure 8 shows the diffusion coefficient of carbon dioxide determined for two different types of closures with the new method according to Figure 3. Each measurement is the average of seven individual measurements under the same operating conditions (except cotton, 100 mL, MgCl$_2$ is the average of six measurements). The error bars indicate the standard deviation for each experimental condition. The maximum standard deviation is 11% of the average, indicating a good reproducibility of the new method presented.

For those experiments in which cotton plugs were investigated, shake flasks of different nominal volumes and with different types of salt solutions were employed. As a result, an average $D_{CO2} = 0.1 \text{ cm}^2/\text{s}$ was found for all combinations of flask nominal volume—salt solution. It is noteworthy that the same value is obtained, irrespective of the salt species employed, underlining the applicability of the method presented in this paper.
For paper plugs however, the value of $D_{CO_2} = 0.09 \text{ cm}^2/\text{s}$ is somewhat lower than for cotton plugs, indicating a slightly higher mass transfer resistance for this industrially produced sterile closure type. The present values may now be used to assess the mass transfer resistance of the investigated sterile closure types. Thanks to these results, a rational design of the operating conditions of shake flasks may be performed with respect to the mass transfer resistance between the environment and the liquid bulk.

**Conclusion**

In this paper a new method is presented for the determination of the mass transfer resistance of sterile closures in shake flasks. It does not require any special equipment other than a balance and can be performed by any laboratory staff. The method is inexpensive, easy, and quick to apply, generating reproducible data for the design of experimental conditions for shake flask experiments.

**Nomenclature**

- $A$: cross-sectional area of the flask neck ($\text{m}^2$)
- $D_i$: diffusion coefficient of the gas component $i$ in a multicomponent mixture ($\text{m}^2/\text{s}$)
- $D_{CO_2}$: diffusion coefficient of carbon dioxide in a multicomponent mixture ($\text{m}^2/\text{s}$)
- $D_{N_2}$: diffusion coefficient of nitrogen in a multicomponent mixture ($\text{m}^2/\text{s}$)
- $D_{av}$: average diffusion coefficient of oxygen ($\text{m}^2/\text{s}$)
- $D_i$: diffusion coefficient in binary gas mixture ($\text{m}^2/\text{s}$)
- $D_{CO_2/N_2}$: diffusion coefficient of carbon dioxide in a binary mixture with nitrogen ($\text{m}^2/\text{s}$)
- $D_{CO_2/O_2}$: diffusion coefficient of carbon dioxide in a binary mixture with oxygen ($\text{m}^2/\text{s}$)
- $D_{CO_2/H_2O}$: diffusion coefficient of carbon dioxide in a binary mixture with water vapor ($\text{m}^2/\text{s}$)
- $\varepsilon$: error tolerance
- $ER$: evaporation rate from shake flask ($\text{m}^3/\text{h}$)
- $ER'$: evaporation rate from second shake flask ($\text{m}^3/\text{h}$)
- $F$: geometry factor ($\text{m}$)
- $F'$: geometry factor of the second shake flasks ($\text{m}$)
- $h$: height coordinate of the closure ($\text{m}$)
- $H$: height of the closure ($\text{m}$)
- $m_0$: mass of the shake flask at the beginning of the experiment ($\text{kg}$)
- $m_1$: mass of the shake flask at the end of the experiment ($\text{kg}$)
- $p$: total pressure ($\text{bar}$)
- $p_i$: partial pressure of the component $i$ ($\text{bar}$)
- $p_{O_2}$: partial pressure of oxygen ($\text{bar}$)
- $p_{CO_2}$: partial pressure of carbon dioxide ($\text{bar}$)
- $p_{O_2\text{, in}}$: partial pressure of oxygen in the flask head space ($\text{bar}$)
- $p_{O_2\text{, out}}$: partial pressure of oxygen in the surrounding environment ($\text{bar}$)
- $q_i$: cross-area specific volumetric flow rate of the component $i$ across the sterile closure ($\text{m}^3/\text{s}$)
- $Q_i$: total volumetric flow of the gas component $i$ ($\text{m}^3/\text{s}$)
- $Q$: total volumetric flow of nitrogen ($\text{m}^3/\text{s}$)
- $RH_{in}$: measured relative humidity of the headspace of the flask ($\%$)
- $RH_{in}$: measured relative humidity of the headspace of the second flask ($\%$)
- $RH_{in\text{, calc}}$: calculated relative humidity of the headspace of the flask ($\%$)
- $RH_{in\text{, calc}}$: calculated relative humidity of the headspace of the second flask ($\%$)
- $RH_{out}$: average relative humidity in the surrounding environment ($\%$)
- $RQ$: respiratory coefficient
- $t$: experimental time ($\text{s}$)
- $T$: temperature ($\text{K}$)
- $w$: velocity ($\text{m/s}$)
- $V_G$: gas volume of the headspace of the flask ($\text{m}^3$)

**References**


