

Keywords

- LTSF sterilisation process
- Biological indicators for LTSF sterilisation processes
- Resistometers for LTSF processes
- Resistance of biological indicators

Reaction Kinetics of the Low-Temperature-Steam-Formaldehyde (LTSF) Sterilisation Process

Characterisation of Biological Indicators for Validation and Routine Monitoring

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The kinetics of the low temperature steam formaldehyde sterilisation process has been described. A method for resistance determination for validation and monitoring of the LTSF process and resistance specifications for suitable biological indicators have been proposed.

Problem Definition

Biological indicators are needed in order to be able to validate sterilisation processes and to conduct routine checks. To determine resistance, the reaction kinetics of the process as well as a reproducible test method tailored to the process, which is comparable with the processes being used in practice, must be known. Under these reproducible conditions, minimum F_0 values must be defined as per ISO 14937 for the respective process in order to ensure that sterilisation of the devices currently used in the healthcare setting complies with the sterilisation assurance level $SAL = 10^{-6}$ (cfu/part) required as per EN 556.

Biological indicators for validation and routine monitoring of LTSF sterilisation processes were already described in DIN 58948-14, using blood serum challenged *B. stearothermophilus* spores strips. This additional treatment for increasing resistance turned out not to be reproducible (1) and was omitted in the new European Standard EN 866-5 which superseded the DIN standard. In the former DIN standard, resistance was measured in a 2 % aqueous formaldehyde solution, while the formaldehyde sterilisation process itself was viewed as taking place in the gas phase. The European Stan-

dard EN 866-5 which has just been published thus calls for resistance determination of biological indicators in the gas phase with 10 mg formaldehyde/l at 70 °C.

A resistometer for these measurements has been neither described hitherto in the EN Standard nor was it possible to find any allusions to it in the literature.

The resistometers (2, 3) featured hitherto in the literature inject the necessary formaldehyde quantities in an aqueous solution into a thermostat-monitored sterilisation chamber and vaporise this solution in order to generate the necessary steam and formaldehyde partial pressure values in the chamber. Using this method, neither we nor other authors were able to maintain constant formaldehyde concentrations in the gas phase because the formaldehyde changes very rapidly into condensate droplets on the wall and thus becomes depleted in the gas phase.

Construction and Operation of a Resistometer as per EN 866-5

By presenting a formaldehyde-water solution in a temperature-monitored glass flask we produced a constant concentration of formaldehyde and steam in the gas phase which is determined by its partial pressure over the solution.

The temperature of the double sheath of a glass flask with a capacity of approx. 2 litres was regulated with a water bath. The flask was equipped with a gas-tight stirrer and connected to a vacuum pump. The biological indicator samples to be measured were secured to the hook of a ground-in stopper and placed in the upper section of the flask.

The reaction chamber was evacuated to initially 100 mbar. Then 311 mbar steam

(70 °C) was introduced into the chamber. These two steps were repeated four times to remove air from the chamber.

Then approx. 500 ml of temperature-regulated formaldehyde-water solution was drawn off by opening a glass cock which was connected to a formaldehyde-water storage container. By agitating the solution, the steam and formaldehyde partial pressure was generated within one minute and remained constant for the entire measurement period. Formaldehyde consumption was supplied continually from the liquid phase. There was no fear of formaldehyde depletion because the concentration between liquid and gas phase differed by about the factor 10,000. At the end of the test, the supply line to the vacuum pump was detached, the formaldehyde solution was removed from the glass flask and evacuated once again in order to remove residual quantities of formaldehyde from the sample. Then the sample was evaluated as described in section "Evaluation of Biological Indicators".

There was widespread concordance between the concentration values measured by us (table 1) and data published by Walker (4).

Using this method, it was possible to achieve well-reproducible results with the temperatures given above. At the same time table 1 illustrates that at equilibrium large quantities of formaldehyde are present

Temperature [°C]	50	60	70
Formaldehyde concentration [mol/l]	10,5	5,2	2,9
[Weight %]	29	15	8,5

Table 1 Formaldehyde concentration required for production of 10 mg formaldehyde/l in the gas phase at partial pressure equilibrium.

in the liquid. The concentration of formaldehyde in the liquid and gas phase has a ratio of approx. 1: 10,000. Hence a large quantity of formaldehyde from the gas phase is changed into a few water condensate droplets. If only the pre-calculated quantity of formaldehyde is injected, there is depletion of the formaldehyde concentration in the gas phase and – due to the process – no reproducible formaldehyde concentrations are preserved in the gas phase

Determination of the Resistance of Biological Indicators in the Gas Phase

Tests to determine resistance were conducted in the resistometer described. The half-logarithmic survival curves were not linear and show inflection after a plateau phase which was not reproducible.

Measurements of formaldehyde load on biological indicator carriers over time showed that a formaldehyde concentration up to the equilibrium concentration in line with the formaldehyde concentration is formed in the solution. This diffusion process is a time-consuming reaction lasting for 10 – 40 min and greatly depends on the ambient conditions, e.g. the concentration increases more quickly on using a ventilator in the resistometer or on using naked biological indicators without glassine packaging.

The plateau measured at the beginning of the survival curve is determined by this difficult-to-reproduce diffusion process. Only on reaching the equilibrium concentration on the biological indicator can reproducible kill velocities be expected and also measured.

Reaction Medium

The measurements outlined in the last section confirmed what was already published by Horn (5), namely that kill is not mediated by formaldehyde in the gas phase but rather takes place in a condensate film covering all surfaces and enclosing the items being sterilised and containing around 10,000-fold higher formaldehyde volume concentration than in the gas phase (Cf. section "Construction and Operation of a Resistometer").

These observations give rise to the question as to whether it is of benefit to use a resistometer that works in the gas

phase, since the reaction itself takes place in the liquid phase, i.e. in a condensate film, and hence reproducibility in the gas phase cannot in principle be guaranteed.

Therefore all further measurements for determination of the reaction kinetics were conducted in the liquid phase with aqueous formaldehyde solutions.

The formaldehyde solutions used were produced by dissolution of paraformaldehyde (Merck) in water. No methanol was added to stabilise the solution since the formaldehyde vapour pressure over an aqueous formaldehyde solution and the content of free, non-hydrated formaldehyde in the solution are affected also by the methanol content (4).

Via the equilibrium partial pressures, it was possible to establish a correlation between the concentrations in the gas phase and the concentrations in the liquid phase.

Critics complain that resistance measurements, as proposed, are not conducted under the same conditions as those prevailing in customary LTSF processes in the gas phase. A counter argument applicable here is that:

1. the microbicidal effect is indeed generated in the liquid condensate phase, and
2. almost all real LTSF processes are conducted under non-equilibrium conditions. Hence the formaldehyde concentrations formed via the diffusion process in the condensate film are indefinite.

The method proposed here in the liquid phase, which actually comes closest to the LTSF process, permits reproducible kinetic measurements.

ing to them may inhibit germination. The biological indicators must be freed of formaldehyde before incubation. This can be accomplished by addition of histidine or cysteine to the nutrient solution or by preliminary neutralisation with sodium sulphite solution (Na₂SO₃).

We used a method devised by the Robert Koch Institute (RKI) Berlin and modified by us:

1. Immersion in 2 % Na₂SO₃ solution for 10 min
2. 1 h heat activation at 90 – 93 °C
3. Incubation at 55 °C for 5 days

Without heat activation, germination of *B. stearothermophilus* can take as long as 14 days.

Reaction Kinetics

Spicher and Peters (7, 8) already noted that the microbial kill velocity in formaldehyde solutions is a function of temperature, concentration and microbial count. We conducted measurements in 1-, 2- and 3-molar formaldehyde solutions at 50 and 70 °C (Table 2).

In the course of these measurements we noted that the kill velocity increases somewhat proportional to the formaldehyde concentration. The elucidated z-value is between 21 and 24 °C. We did not elucidate whether this scattering is attributable to measurement errors or to the complexity of the reaction.

Unlike the steam sterilisation process, there is no reaction kinetics of the first order manifest here but rather of the second order.

$$-\frac{dN}{dt} = k \times [FA]^n \times N \quad (1)$$

The measured exponent n for the formaldehyde concentration has the value n = 1.3 at 50 °C and the value n = 1.0 for 70 °C. Peters and Spicher found an exponent of 1.05

Evaluation of Biological Indicators

When biological indicators are withdrawn from the reaction container, be it from the gas or liquid phase, formaldehyde adher-

Temperature [°C]	FA exponent n	Formaldehyde concentration [M]	1	2	3
50	1,3	D _{FA} [min]	27,1	11,8	6,0
		D [M min]	27,1	23,6	18
70	1,0	D _{FA} [min]	3,0	1,5	0,9
		D [M min]	3,0	3,0	2,7
		z-value [°C]	21	23	24

Table 2 D- and z-values for different temperatures and formaldehyde concentrations

± 0.1 in the temperature range 50 °C – 80 °C. Within the measurement precision this concurs with a reaction kinetics of the second order and a linear correlation between formaldehyde concentration and kill.

Assuming that n = 1, the integral of the equation (1) is within the limits of N₀ to N:

$$\lg \frac{N_0}{N} = \lg N_0 - \lg N = \frac{1}{D} \times [FA] \times t = IF \quad (2)$$

IF = Inactivation factor (number of decimal reduction levels)

N₀ = Initial microbial count [cfu/part]

N = Residual microbial count after sterilisation time t [cfu/part]

t = Sterilisation time [min]

M = Molar concentration [mol/l]

[FA] = Formaldehyde concentration [M] in the condensate or in the presented solution

k' = $\frac{1}{D}$ = Reaction velocity constant $\frac{1}{M \times \text{min}}$

D_{70°C, 1M FA} = Decimal reduction factor measured at 70°C and 1 M FA [min]

n = Exponent of FA concentration (dimensionless)

D_{FA} = D-value referred to a certain [FA] [min]

Due to the reaction kinetics of the second order, the D-value has the dimension [M min]. Although the D-value is independent of the formaldehyde concentration, the reaction velocity increases with increasing formaldehyde concentration.

Hence the quotient $\frac{D}{[FA]} = D_{FA}$ is comparable with the D-value for reaction kinetics of the first order, i.e. for example on doubling the formaldehyde concentration the quotient, and hence the reaction time, is halved.

New Standard Proposal

Based on the experience gathered, we propose for discussion the following values when EN 866-5 is revised at the time of compilation of a new ISO EN standard.

1. Resistometer: 1 M formaldehyde solution at 60°C
2. Incubation as per section „Evaluation of Biological Indicators“
3. Biological indicators
Microbe: *B. stearothermophilus*

for N ₀ =10 ⁶ cfu/part	dimension	SAL	IF	for 1 M FA		for 2 M FA	
				min	max	min	max
D	M _{min}			5	6	2,5	3
F _{BIO}	M _{min}			30	36	15	18
survival time	min	10 ²	4	20	24	10	12
kill time	min	10 ⁻⁴	10	50	60	25	30
window	min	–	–	20	60	10	30
				growth	kill	growth	kill

Table 3 Limit values of the kill-survival window at 60°C

for N ₀ =10 ⁶ cfu/part	dimension	SAL	IF	for 1 M FA		for 2 M FA	
				min	max	min	max
D	M _{min}			1,7	2,1	0,85	1,05
F _{BIO}	M _{min}			10,2	12,6	5,1	6,3
survival time	min	10 ²	4	6,8	8,4	3,4	4,2
kill time	min	10 ⁻⁴	10	17	21	8,5	10,5
window	min	–	–	6,8	21	3,4	10,5
				growth	kill	growth	kill

Table 4 Limit values of the kill-survival window at 70°C

Germ carrier: glass wool
Population: 10⁵ – 10⁷ cfu/strip

4. Resistance:

The total resistance of a biological indicator is not determined only by the microbial count but rather also by the resistance (D-value) of the microbe employed. The total resistance of the biological indicator is thus determined by its F_{BIO} value:

$$F_{BIO, 60^\circ C, 1M FA} = \frac{D}{[FA]} \times \lg N = \frac{\geq 30 \leq 36}{[FA]} \text{ [min]} \quad (3)$$

This corresponds to a D-value window of

$$D_{60^\circ C, 1M FA} \geq 4 \leq 7 \text{ [M min]}$$

For a defined formaldehyde concentration and temperature, the F_{BIO} value specifies for how long sterilisation must be conducted so as to reduce the microbial count on average to the value 1 [cfu/strip].

Correspondingly adapted values apply for other formaldehyde concentrations. The proposed kill-survival window at 60 °C with an exposure time of 60 min corresponds somewhat to the LTSF sterilisation processes currently used.

On using the z-value of 22 °C taken from table 2 as the mean value of 1 and 2 M formaldehyde concentration, the limit values as given in table 4 are obtained for biological indicators on increasing the temperature to 70 °C.

Correspondingly adapted values apply for other formaldehyde concentrations.

The proposed values should be checked with different temperatures and formaldehyde concentrations as per the requirements of ISO 14937.

Conclusions

1. The proposed process can serve as a basis for resistance determination of biological indicators for LTSF sterilisation processes.

2. The elucidated highly time-dependent diffusion processes of formaldehyde from the gas phase to the condensate film on the surface of the items to be sterilised calls for, in addition to monitoring of the reaction kinetics described here, precise monitoring of the penetration kinetics of formaldehyde to all inner and outer surfaces of the products to be sterilised. Monitoring of the penetration kinetics of the sterilisation gas on the inner surfaces can be effected only with a suitable process challenge device (PCD) and must especially be taken into account for validation and routine monitoring.

3. Therefore generation of a standard for validation of this process is necessary.*

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