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Application of optimal experimental design concept to improve the estimation of model parameters in microbial thermal inactivation kinetics

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ABSTRACT

The estimation of model parameters with high precision is of major importance in mathematical predictions. If a mathematical model is properly chosen and if the primary objective is to improve parameter estimation, underlying statistical theories can be applied. Precision increases with the number of experimental points. However, and in many situations, maximum precision is attained when sampling consists of replicates of specific experimental points. Experimental conditions can be optimized using the *D*-optimal design concept based on minimization of the generalized variance of the parameter estimates.

The objective of this work was to use this methodology for the design of experiments for microbial inactivation processes described by a Gompertz-based model under isothermal and non-isothermal conditions. The application of *D*-optimal design concept considerably improved parameters precision, when compared to the commonly used heuristic designs.

1. Introduction

Predictive microbiology is gaining considerable importance in the food processing domain, particularly in the design of efficient and safe inactivation treatments. This terminology designates the use of mathematical models describing microbial kinetics. Those models should predict accurately and precisely the microbial behaviour, which depends on the adequacy of the model and the quality of the estimated parameters. If a mathematical model structure is properly chosen (assessed by its adequacy in data fitting), the reliable estimation of model parameters is the concern. In order to improve parameter estimation (i.e. accurate and precise estimates; low correlations between estimates, and narrow confidence intervals/regions), the selection of the most informative pattern of experimental points is crucial.

Fisher introduced the concept of Experimental Design in the 1920s, inspired by problems in the field of agricultural experiments (Fisher, 1922). He concluded that experimental data, the skeleton of parameter estimation, should be informative and "no sophisticated and complex techniques of statistical data analysis can provide good results if experimental data are poor *a priori*" (Box et al., 1978). Bates and Watts (1988) stressed that the prime importance of experimental design arises from the fact that the information content of the data is established when the experiment is performed, and no amount of sensitive analysis can recover information which is not present in the data. Experimental design has been extensively studied since the 1980s in diverse fields seeking to optimize experimental conditions with respect to parameter estimation. Brandão and Oliveira (1997), when studying kinetics of mass transfer, used the *D*-optimal experimental design as an optimal criterion minimizing the parameters' variance. Cunha et al. (1998) applied this criterion to analyse systems described by the Weibull probabilistic model, Azevedo et al. (1998) for the joint estimation of diffusion and external mass transfer coefficients, and Brandão et al. (2001) for the estimation of mass transfer diffusion parameters in food processing.

The precision of parameter estimates increases with the sampling size. However, and in many situations, when the replicates of a number of experimental points is equal to the number of model parameters, a maximum precision is attained. One should bear in mind that this design criterion assumes that the model is known and that the objective is the estimation of model parameters with increased precision.

There are other alternative criteria that can be used, depending on the objective of the design. Steinberg and Hunter (1984)

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Nomenclature

A_{Ratk_i}	parameter i of the Ratkowsky model that relates L with T (i = 1, 2)
e	Euler's number (base of the natural logarithms)
E_a	activation energy (J mol^{-1})
i	given experimental condition (i = 1, ...n)
j	given model parameter (j = 1, ...p)
J	Jacobian matrix; matrix of the derivatives of the model with respect to the parameters
k	inactivation rate (s^{-1}; min^{-1})
L	Shoulder parameter (s; min)
n	number of experimental points or sampling conditions
N	microbial cell density (CFU ml^{-1})
p	number of model parameters
R	gas constant (8.314 J mol^{-1} K^{-1})
s	standard deviation
SSR	sum of squares of the residuals
t	time (s; min)
t'	dummy variable
t_i	time at experimental condition i (min; s)
$t_{\nu,\alpha/2}$	random variable following a t-Student distribution with ν (=n–p) degrees of freedom, leaving an area of (α/2) to the right
T	temperature (K; °C)
y	logarithm of the microbial cell density normalized in relation to the initial value

Greek symbols

α	significance level
β	temperature increase rate (°C s^{-1}; °C min^{-1})
Δ	determinant of the matrix [$\mathbf{J^TJ}$]
ε	efficiency of the heuristic experimental design
θ_j	vector of model parameters (j = 1, ...p)

Subscripts

ave	relative to average value
exp	relative to experimental value
heu	relative to heuristic sampling
isot-range	relative to isothermal conditions and range of temperatures
isot-single	relative to isothermal conditions and single temperature
max	maximum value
non-isot	relative to non-isothermal conditions
opt	relative to optimal condition
ref	reference value
res	residual value
0	initial value

Superscripts

T	transpose of a matrix

summarised the most common criteria in linear situations. Despite its advantages in the design of optimal experiments, this concept is not commonly utilized by food microbiologists. Grijspeerdt and Vanrolleghem (1999) and Versyck et al. (1999) introduced optimal experimental design in predictive microbiology and showed that if an optimal experimental design criterion is used, experimental data enable unique model parameter estimation, being more precise than the one obtained from classical heuristic sampling schemes.

Isothermal and non-isothermal experiments can be planned according to the *D*-optimality criterion. At constant temperature, this criterion allows the definition of sampling times and temperature conditions while under non-isothermal conditions, the procedure allows the selection of a heating rate within a desirable temperature range. Bernaerts et al. (2002) presented a case study in which non-isothermal temperature profiles were optimized to establish accurate estimation of *Escherichia coli* growth. Bernaerts et al. (2005), Balsa-Canto et al. (2008) and Van Derlinden et al. (2008, 2010) also developed optimal experimental designs applied to the field of predictive microbiology.

The objective of our work was to define optimal experimental conditions according to *D*-optimal concept, for microbial thermal inactivation processes showing a sigmoidal behaviour, *i.e.*, an initial lag phase (or shoulder), followed by a maximum inactivation rate period, tending to a tail residual population. A Gompertz-based model was used to describe this behaviour. *D*-optimal design was applied aiming at improving the estimation of kinetic parameters. Isothermal and non-isothermal conditions were considered. Comparisons with commonly used heuristic designs were also assessed.

2. Mathematical considerations

2.1. The models

2.1.1. Single temperature

According to Gil et al. (2011), a Gompertz-modified model can be used to describe sigmoidal microbial inactivation, under isothermal conditions:

$$y_{\text{isot-single}}(t) = \log\left(\frac{N}{N_0}\right) = \log\left(\frac{N_{\text{res}}}{N_0}\right)\exp\left(-\exp\left(-\frac{k_{\max}e}{\log\left(\frac{N_{\text{res}}}{N_0}\right)}(L-t)+1\right)\right) \quad (1)$$

Herein $y_{\text{isot-single}}$ represents the microbial cell density: logarithm of the microbial load (N) at a certain process time (t), normalized in relation to the initial value (N_0); N_{res} is the residual microbial load. Eq. (1) is a two-parameter model, where L is the shoulder parameter (initial lag) and $k_{\max}$ the maximum inactivation rate. N_0 is not considered a model parameter and should be obtained experimentally.

2.1.2. Range of temperatures

If a range of temperatures is considered, the temperature dependence of $k_{\max}$ and L may be included in the previous model. Assuming that an Arrhenius relationship and a Ratkowsky equation can be used to describe such dependences (Ratkowsky et al., 1982), the Gompertz-based model is transformed into a four-parameter model as follows:

$$y_{\text{isot-range}}(t) = \log\left(\frac{N_{\text{res}}}{N_0}\right)\exp\left(-\exp\left(\frac{\left(-k_{\text{ref}}\exp\left(-\frac{E_a}{R}\left(\frac{1}{T}-\frac{1}{T_{\text{ref}}}\right)\right)\right)e}{\log\left(\frac{N_{\text{res}}}{N_0}\right)}\left(\left(A_{\text{Ratk}_1}(T-A_{\text{Ratk}_2})\right)^2-t\right)+1\right)\right) \quad (2)$$

where the parameters are k_{ref} (the inactivation rate at a finite reference temperature T_{ref}), E_a (the process activation energy), and A_{Ratk_1} and A_{Ratk_2} (the Ratkowsky equation parameters); R is the universal gas constant.

2.1.3. Time-varying temperature conditions

For non-isothermal conditions, the following time-integrated expression of Eq. (1) can be used (Gil et al., 2006, 2011):

$$y_{non-isot} = \int_0^t \left[-k_{max}(T) e \exp\left(-\frac{k_{max}(T) e}{\log\left(\frac{N_{res}}{N_0}\right)} (L(T) - t') + 1 \right) \exp\left(-\exp\left(-\frac{k_{max}(T) e}{\log\left(\frac{N_{res}}{N_0}\right)} (L(T) - t') + 1 \right) \right) \right] dt' \quad (3)$$

If the Arrhenius and Ratkowsky expressions are included in the model to describe respectively the temperature dependence of k_{max} and L, and if the temperature history $T(t)$ is known, $y_{non\text{-}isot}$ at a particular time can be given by:

$$y_{non-isot} = \int_0^t \left[\left(k_{ref} \exp\left(-\frac{E_a}{R}\left(\frac{1}{T(t)} - \frac{1}{T_{ref}}\right)\right)\right) e \exp\left(\frac{\left(k_{ref} \exp\left(-\frac{E_a}{R}\left(\frac{1}{T(t)} - \frac{1}{T_{ref}}\right)\right)\right) e}{\log\left(\frac{N_{res}}{N_0}\right)} \left(\left[A_{Ratk_1} (T(t) - A_{Ratk_2}) \right]^2 - t' \right) + 1 \right) \times \exp\left(-\exp\left(\frac{\left(k_{ref} \exp\left(-\frac{E_a}{R}\left(\frac{1}{T(t)} - \frac{1}{T_{ref}}\right)\right)\right) e}{\log\left(\frac{N_{res}}{N_0}\right)} \left(\left[A_{Ratk_1} (T(t) - A_{Ratk_2}) \right]^2 - t' \right) + 1 \right) \right) \right] dt' \quad (4)$$

The model parameters are therefore k_{ref}, E_a, A_{Ratk_1} and A_{Ratk_2}.

2.2. Design criterion

Experiments can be designed according to a *D*-optimal criterion. This is equivalent to minimize the parameters' variance, which mathematically corresponds to the minimisation of the absolute value of the determinant of the variance-covariance matrix $|\mathbf{J}^T\mathbf{J}|^{-1}$, or maximization of $|\mathbf{J}^T\mathbf{J}|$ (Atkinson, 1982). The elements of the $\mathbf{J}$ matrix are the sensitivities of the mathematical model used to describe the process (Eqs. (1), (2), (4); θ_j, $j = 1, \ldots p$) evaluated at all experimental conditions, i:

$$\mathbf{J} = \begin{bmatrix} \left.\frac{\partial y}{\partial \theta_1}\right|_{i=1} & \cdots & \left.\frac{\partial y}{\partial \theta_p}\right|_{i=1} \\ \vdots & & \vdots \\ \left.\frac{\partial y}{\partial \theta_1}\right|_{i=n} & \cdots & \left.\frac{\partial y}{\partial \theta_p}\right|_{i=n} \end{bmatrix} \quad (5)$$

If the number of model parameters (p) equals the number of experimental conditions (n), $\mathbf{J}$ is a square matrix (pxp).

The definition of the *D*-optimal experimental design requires preliminary parameters estimates. This is paradoxical, because the purpose of the experimental design is to obtain such estimates with increased precision. However, Brandão et al. (2001), when studying optimal experimental conditions in mass transfer kinetics, reported that *D*-optimal design is robust to considerable high variations of those estimates. Therefore, the experimental design definition is not compromised in such circumstances.

3. Methodology

3.1. Isothermal conditions

3.1.1. Single temperature

Considering a single isothermal experiment, microbial inactivation is described by Eq. (1) and, consequently, two parameters should be estimated: k_{max} and L. According to the simplest design criterion, maximum precision of the parameters is achieved if two sampling times are planned ($n = 2$). The expression of the determinant becomes:

$$\Delta = \begin{vmatrix} \sum_{i=1}^{n} \left(\frac{\partial y_{isot-single}}{\partial k_{max}} \right)_i^2 & \sum_{i=1}^{n} \left(\frac{\partial y_{isot-single}}{\partial k_{max}} \times \frac{\partial y_{isot-single}}{\partial L} \right)_i \\ \sum_{i=1}^{n} \left(\frac{\partial y_{isot-single}}{\partial L} \times \frac{\partial y_{isot-single}}{\partial k_{max}} \right)_i & \sum_{i=1}^{n} \left(\frac{\partial y_{isot-single}}{\partial L} \right)_i^2 \end{vmatrix} = \sum_{i=1}^{n} \left(\frac{\partial y_{isot-single}}{\partial k_{max}} \right)_i^2 \sum_{i=1}^{n} \left(\frac{\partial y_{isot-single}}{\partial L} \right)_i^2 - \sum_{i=1}^{n} \left(\frac{\partial y_{isot-single}}{\partial k_{max}} \times \frac{\partial y_{isot-single}}{\partial L} \right)_i^2 \quad (6)$$

in which the sampling condition i corresponds to a sampling time t_i for one experiment at a temperature T_i. Preliminary *L. innocua* thermal inactivation experiments were performed by Miller et al. (2009) at 52.5, 55.0, 57.5, 60.0, 62.5 and 65.0 °C. These experimental data, fitted with the Gompertz-inspired model, provided initial estimates of k_{max} and L required for calculations (Table 1; Miller et al., 2009). These parameters were estimated on the basis of a heuristic sampling, which means that sampling points were spaced on the time scale with approximately equal intervals (Miller et al., 2009). Residual microbial density, *i.e.* $\log(N_{res}/N_0)$, was assumed to be independent of temperature. The value considered was −10 in all cases,

Table 1
Parameters used in definition of *D*-optimal experimental design and corresponding optimal sampling conditions, using a single temperature.

Variables			Optimal sampling			
T (°C)	k_{max} (min^{-1})	L (min)	t_{1-opt} (min)	$\log(N/N_0)_{t1-opt}$	t_{2-opt} (min)	$\log(N/N_0)_{t2-opt}$
52.5	4.04×10^{-2}	69.06	91.8	−0.962	162.1	−3.516
55.0	7.56×10^{-2}	39.58	51.8	−0.962	89.3	−3.516
57.5	1.41×10^{-1}	10.82	17.4	−0.962	37.5	−3.516
60.0	4.52×10^{-1}	6.06	8.1	−0.962	14.4	−3.516
62.5	1.14	0.68	1.5	−0.962	4.0	−3.516
65.0	2.18	0.03	0.5	−0.962	1.8	−3.516

i.e. an average estimation obtained by Miller et al. (2009) from isothermal experiments.

The two optimal sampling times (t_{1-opt} and t_{2-opt}) that minimize the reciprocal of Δ (Eq. (6)) were calculated numerically, using the Solver application available in Microsoft Excel (2010 Microsoft Corporation, USA) and choosing the simplex method for function minimisation (Nelder and Mead, 1965). The corresponding microbial cell density at times t_{1-opt} and t_{2-opt} were calculated using Eq. (1).

3.1.2. Range of temperatures

If a range of temperatures is considered, inactivation behaviour is described by Eq. (2) and four parameters should be estimated: k_{ref}, E_a (of the Arrhenius model), A_{Ratk1} and A_{Ratk2} (of the Ratkowsky model). If four sampling conditions (*i.e.* four combinations of temperature/sampling time) are planned ($n = 4$), the determinant becomes:

$$\Delta = \begin{vmatrix} \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial k_{ref}}\right)_i^2 & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial k_{ref}}\times\frac{\partial y_{isot-range}}{\partial E_a}\right)_i & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial k_{ref}}\times\frac{\partial y_{isot-range}}{\partial A_{Ratk_1}}\right)_i & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial k_{ref}}\times\frac{\partial y_{isot-range}}{\partial A_{Ratk_2}}\right)_i \\ \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial E_a}\times\frac{\partial y_{isot-range}}{\partial k_{ref}}\right)_i & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial E_a}\right)_i^2 & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial E_a}\times\frac{\partial y_{isot-range}}{\partial A_{Ratk_1}}\right)_i & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial E_a}\times\frac{\partial y_{isot-range}}{\partial A_{Ratk_2}}\right)_i \\ \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial A_{Ratk_1}}\times\frac{\partial y_{isot-range}}{\partial k_{ref}}\right)_i & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial A_{Ratk_1}}\times\frac{\partial y_{isot-range}}{\partial E_a}\right)_i & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial A_{Ratk_1}}\right)_i^2 & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial A_{Ratk_1}}\times\frac{\partial y_{isot-range}}{\partial A_{Ratk_2}}\right)_i \\ \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial A_{Ratk_2}}\times\frac{\partial y_{isot-range}}{\partial k_{ref}}\right)_i & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial A_{Ratk_2}}\times\frac{\partial y_{isot-range}}{\partial E_a}\right)_i & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial A_{Ratk_2}}\times\frac{\partial y_{isot-range}}{\partial A_{Ratk_1}}\right)_i & \sum_{i=1}^{n}\left(\frac{\partial y_{isot-range}}{\partial A_{Ratk_2}}\right)_i^2 \end{vmatrix} \quad (7)$$

in which the sampling condition i corresponds to a sampling time t_i for one experiment at a temperature T_i. Six temperatures within a range were considered: 52.5, 55.0, 57.5, 60.0, 62.5 and 65.0 °C. Preliminary estimates of k_{ref}, E_a, A_{Ratk1} and A_{Ratk2} required for calculations were assumed to be $0.29\ min^{-1}$, $3.34 \times 10^5\ J\ mol^{-1}$, $0.62\ K^{-1}\ min^{0.5}$, 337.1 K, respectively, which are representative of *Listeria innocua* inactivation in the temperature conditions considered (Gil, 2009). The reference temperature (T_{ref}) was assumed to be 331.9 K (*i.e.* middle value of the range 52.5–65.0 °C; Cohen et al., 1994). Residual microbial density, *i.e.* $\log(N_{res}/N_0)$, was assumed to be −10 (reasons already outlined above).

The four optimal sampling conditions (i.e. four combinations of optimal temperature/optimal sampling, T_{i-opt}/t_{i-opt}, $i = 1, \ldots 4$) that minimize the reciprocal of Δ (Eq. (7)) were calculated numerically, as described before.

3.2. Time-varying temperature conditions

In this case, the elements of the **J** matrix are the partial derivatives of the mathematical model used to describe the non-isothermal process (Eq. (4)) with respect to each parameter (k_{ref}, E_a, A_{Ratk1} and A_{Ratk2}), evaluated at four sets of sampling conditions (*i.e.* temperature/sampling time). Assuming linear temperature profiles within the temperature range 18.8–65.5 °C (*i.e.* $T = T_0 + \beta t$, where T_0 is the initial temperature and β is the heating rate), expressions for the partial derivatives of the model with respect to the kinetic parameters were obtained. The implications of using a linear heating rate assumption is that temperatures have the same weight in the calculation of the kinetic parameters, which is an important point to be taken into consideration.

Preliminary estimates of k_{ref}, E_a, A_{Ratk_1} and A_{Ratk_2} required for calculations were the ones obtained for non-isothermal inactivation of *L. innocua*, assuming a process in which temperature follows a rapid heating of $2.5\ °C\ min^{-1}$ during 19 min, and then holds at 65.5 °C for the remaining process time (Gil, 2009): $k_{ref} = 0.51\ min^{-1}$, $E_a = 9.84 \times 10^4\ J\ mol^{-1}$, $A_{Ratk_1} = 0.02\ K^{-1}\ min^{0.5}$ and $A_{Ratk_2} = 357.8$ K; T_{ref} was assumed to be 331.9 K (i.e., middle value of the range 52.5–65.0 °C) and $\log(N_{res}/N_0)$ was considered independent of temperature and equal to −18 (asymptotic value considerable low; equivalent to absence of tail population). The four optimal sampling conditions (*i.e.* four optimal sampling times, t_{i-opt}, $i = 1, \ldots 4$) that maximize the absolute value of Δ can be calculated also using Eq. (7), after replacing the corresponding derivatives of non-isothermal model ($y_{non-isot}$; Eq. (4)). A FORTRAN 77 programme (Fortran 5.1, Microsoft Corporation®, 1990) was developed and used for all calculations and optimization procedures based on simplex algorithm for function minimization (Nelder and Mead, 1965).

3.3. Assessment of the precision of the parameters estimates

Precision was assessed by calculating the confidence intervals at $100(1-\alpha)\%$ (Box et al., 1978) of the parameters estimates:

$$\theta_j \pm t_{v,\alpha/2} s_{\theta_j} \quad (8)$$

In the above, $t_{v,\alpha/2}$ is a random variable following a *t*-Student distribution with v ($=n-p$) degrees of freedom, leaving an area of ($\alpha/2$) to the right, and s_{θ_j} is the standard deviation of the parameter estimate:

$$s_{\theta_j} = \sqrt{\frac{SSR}{n-p}\left(\left[\mathbf{J}^T\mathbf{J}\right]^{-1}\right)_{jj}} \quad (9)$$

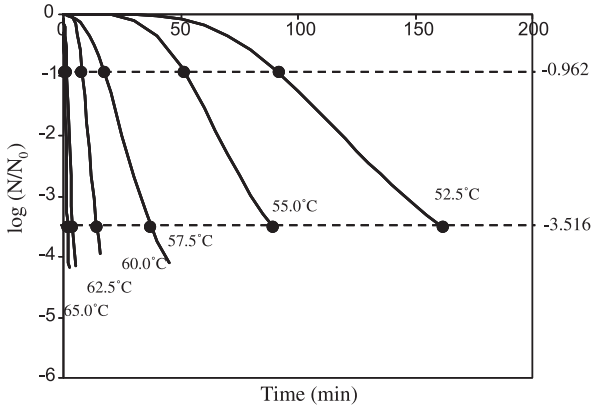

Fig. 1. Computer simulated data of *L. innocua* inactivation under isothermal conditions, assuming parameters presented in Table 1. The dots represent the optimal sampling at each single temperature.

Table 2
Heuristic sampling conditions and design efficiencies calculated on the basis of *D*-optimal design criterion for a single temperature.

	52.5 °C		55.0 °C		57.5 °C		60.0 °C		62.5 °C		65.0 °C	
	t_{heu} (min)	t_{opt} (min)	t_{heu} (min)	t_{opt} (min)	t_{heu} (min)	t_{opt} (min)	t_{heu} (min)	t_{opt} (min)	t_{heu} (min)	t_{opt} (min)	t_{heu} (min)	t_{opt} (min)
	0.0		0.0		0.0		0.0		0.0		0.0	
	0.5		2.5		0.5		0.5		0.1		0.1	
	10.0		5.0		2.0		1.0		0.2		0.3	
	20.0		7.5		4.0		2.0		0.3		0.6	
	30.0		10.0		5.0		3.0		0.4		0.8	0.5
	40.0		12.5		7.0		4.0		0.6		1.2	1.8
	50.0		15.0		9.0		5.0		0.7		1.3	
	60.0		20.0		11.0		6.0		1.0	1.5	1.8	
	70.0	91.8	30.0	51.8	13.0	17.4	7.0	8.1	1.5	4.0	2.0	
	80.0	162.1	40.0	89.3	15.0	37.5	8.0	14.4	2.0		2.2	
	100.0		50.0		20.0		9.0		2.5			
	120.0		60.0		25.0		10.0		3.0			
	140.0		65.0		30.0		11.0		3.6			
	150.0		70.0		35.0		12.0		4.0			
	160.0		75.0		40.0		13.0		4.5			
	170.0		80.0		42.5		14.0		5.0			
	175.0		85.0		45.0		15.0					
	180.0		90.0		47.5		16.0					
Δ_{heu}	1.93×10^{-2}		1.31×10^{-2}		2.47×10^{-2}		2.30×10^{-2}		2.51×10^{-2}		1.72×10^{-2}	
Δ_{opt}		7.37×10^{-2}		7.37×10^{-2}		7.37×10^{-2}		7.37×10^{-2}		5.82×10^{-2}		2.28×10^{-2}
ε (%)	26.2		17.7		33.4		31.2		43.1		75.5	

Table 3
Standard deviation of parameters estimates, using heuristic sampling and *D*-optimal design criteria; single temperature.

T (°C)	Heuristic design		*D*-optimal design	
	$s_{k_{max}}$ (min^{-1})	s_L (min)	$s_{k_{max}}$ (min^{-1})	s_L (min)
52.5	0.09	156.02	0.07	98.15
55.0	0.23	95.22	0.12	52.45
57.5	0.29	37.73	0.23	28.16
60.0	1.07	12.58	0.74	8.78
62.5	2.44	4.50	1.98	3.69
65.0	5.34	2.73	4.78	2.44

where $([\mathbf{J^T J}]^{-1})_{jj}$ is the j^{th} diagonal of the matrix $[\mathbf{J^T J}]^{-1}$ evaluated at each parameter θ_j and SSR is the sum of squares of the residuals, *i.e.*, the sum of squares of the differences between experimental values (y_{exp}) and the ones predicted by the model (y):

$$SSR = \sum_{i=1}^{n} (y_{exp} - y)^2 \quad (10)$$

The precision of the parameters estimates are directly related to the experimental design used, since maximization of $[\mathbf{J^T J}]$ allows the minimization of the variance-covariance of parameters estimates and, consequently, the magnitude of the confidence intervals. Calculations were done with tool packages available in Microsoft Excel (2010 Microsoft Corporation, USA).

4. Results and discussion

4.1. Optimal design of experiments under isothermal conditions

4.1.1. Single temperature

If one single isothermal condition is chosen, the Gompertz model is a two-parameter model. Consequently, the simplest optimal experimental design implies replication of only two sampling times. In this case, results showed that the two sampling times (t_{1-opt} and t_{2-opt}) that minimize the parameters' variance (equivalent to maximization of the determinant in Eq. (6)) were dependent on temperature. The microbial inactivation was calculated by Eq. (1), substituting t_{1-opt} and t_{2-opt} of each temperature. Curiously, optimal experiments consist always on a number of replicates taken at times corresponding to 89.09% (*i.e.* $\log(N/N_0) = -0.96$) and to 99.97% (*i.e.* $\log(N/N_0) = -3.52$) of inactivation (Table 1, Fig. 1). The heuristic experimental design used by Miller et al. (2009) was compared to the *D*-optimal experimental design developed in this work. The efficiency of the heuristic experimental design (ε) was calculated by the ratio between the determinant of the heuristic design (Δ_{heu}) and the one of the *D*-optimal design (Δ_{opt}). Δ_{heu} was calculated for each temperature individually, by

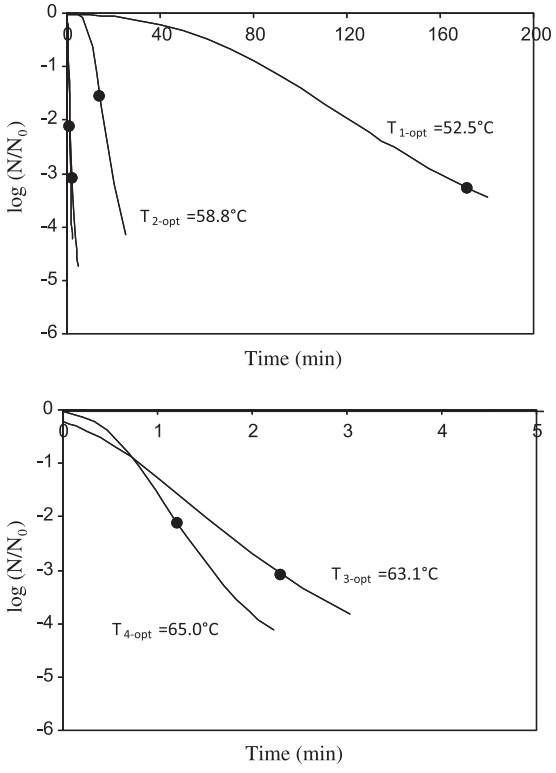

Fig. 2. Computer simulated data of *L. innocua* inactivation under a range of isothermal conditions, assuming $k_{ref} = 0.29$ min^{-1}, $E_a = 3.34 \times 10^5$ J mol^{-1}, $A_{Ratk1} = 0.38$ K^{-1} min$^{0.5}$, $A_{Ratk2} = 337.1$ K and $\log(N_{res}/N_0) = -10$. The dots represent the optimal sampling at each optimal temperature. Lower plot: zoom in on third and fourth sampling conditions.

Table 4
Standard deviation of parameters estimates, using heuristic sampling and *D*-optimal design criteria; range of temperatures and non-isothermal conditions.

Temperature conditions	Parameter estimate	β (°C min^{-1})	s	
			Heuristic design	*D*-optimal design
Range of temperatures	k_{ref} (min^{-1})		2.86×10^{-2}	1.75×10^{-2}
	E_a (J mol^{-1})	–	1.52×10^{4}	8.08×10^{3}
	A_{Ratk_1} (K^{-1} min$^{0.5}$)		5.68×10^{-2}	3.16×10^{-2}
	A_{Ratk_2} (K)		1.46×10^{-1}	8.51×10^{-2}
Non-isothermal conditions	k_{ref} (min^{-1})		2.17×10^{-1}	2.10×10^{-1}
	E_a (J mol^{-1})	2.50	2.59×10^{4}	2.05×10^{4}
	A_{Ratk_1} (K^{-1} min$^{0.5}$)		2.35×10^{-2}	1.00×10^{-2}
	A_{Ratk_2} (K)		3.79×10^{1}	1.37×10^{1}
	k_{ref} (min^{-1})		1.19×10^{-1}	1.17×10^{-1}
	E_a (J mol^{-1})	0.79	1.83×10^{4}	5.64×10^{3}
	A_{Ratk_1} (K^{-1} min$^{0.5}$)		6.89×10^{-3}	3.78×10^{-3}
	A_{Ratk_2} (K)		7.97×10^{0}	6.11×10^{0}

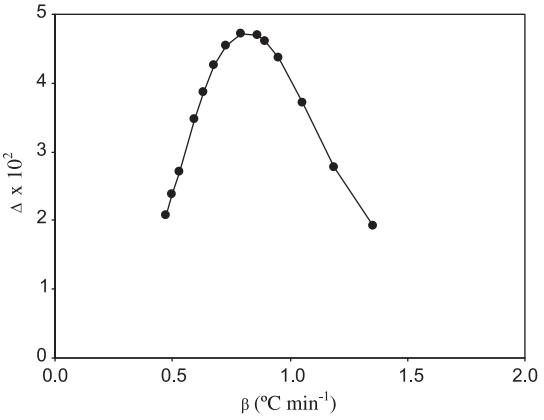

Fig. 3. Influence of the heating rate (β) on the determinant Δ (Eq. (7)), assuming optimal sampling times.

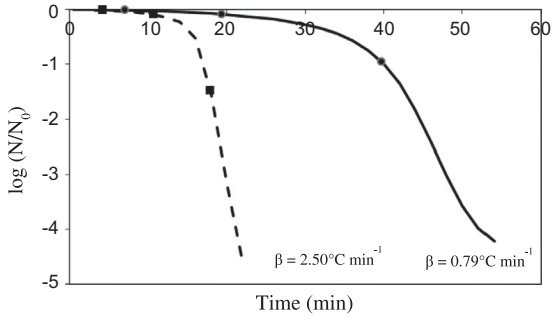

Fig. 4. Computer simulated data of *L. innocua* inactivation under non-isothermal conditions, assuming $k_{ref} = 0.51$ min^{-1}, $E_a = 9.84 \times 10^4$ J mol^{-1}, $A_{Ratk_1} = 0.02$ K^{-1} min$^{0.5}$, $A_{Ratk_2} = 357.8$ K and $\log(N_{res}/N_0) = -18$. The dots represent optimal sampling for $\beta = 0.79$ °C min^{-1} (●) and $\beta = 2.50$ °C min^{-1} (■).

substitution of the n experimental times used by Miller et al. (2009); Δ_{opt} was calculated by substitution of t_{1-opt} and t_{2-opt}, each one replicated $n/2$ times, aiming at considering the same number of experimental points (Table 2).

The efficiency of the heuristic design was 26%, 18%, 33%, 31%, 43% and 76% for the experiments conducted at 52.5, 55.0, 57.5, 60.0, 62.5 and 65.0 °C, respectively. This shows that the heuristic design was not efficient, particularly at the lower temperatures tested, which can be explained by the short extension of the process covered by the sampling times chosen.

If *D*-optimal design had been chosen, the confidence intervals at 95% of k_{max} and L would decrease and precision would be improved for all temperatures tested. The standard deviations of parameters estimates are in Table 3, for both heuristic and *D*-optimal designs.

4.1.2. Range of temperatures

If a range of temperatures is considered, the Gompertz model is a four-parameter model. The simplest optimal experimental design consists of replicates of four experiments conducted within the experimental range: one at each extreme temperature ($T_{1-opt} = T_{min} = 52.5$ °C and $T_{4-opt} = T_{max} = 65.0$ °C), one at the average

Table 5
Definition of *D*-optimal experimental design under non-isothermal conditions: heating rate, sampling times and corresponding $\log(N/N_0)$; evaluation of Δ.

β (°C min^{-1})	Optimal sampling								$\Delta \times 10^2$
	t_{1-opt} (min)	t_{2-opt} (min)	t_{3-opt} (min)	t_{4-opt} (min)	$\log(\frac{N}{N_0})_{t_{1-opt1}}$	$\log(\frac{N}{N_0})_{t_{2-opt}}$	$\log(\frac{N}{N_0})_{t_{3-opt}}$	$\log(\frac{N}{N_0})_{t_{4-opt}}$	
0.48	7.4	19.8	43.3	196.8	−0.015	−0.060	−0.345	−4.690	2.08
0.50	7.4	19.8	43.2	182.4	−0.015	−0.062	−0.370	−4.660	2.38
0.53	7.4	19.7	42.9	204.4	−0.015	−0.064	−0.408	−4.630	2.71
0.59	7.3	19.6	42.4	191.0	−0.015	−0.069	−0.504	−4.570	3.47
0.63	7.3	19.6	41.8	170.4	−0.015	−0.073	−0.569	−4.530	3.87
0.68	7.2	19.5	41.3	189.6	−0.015	−0.077	−0.680	−4.480	4.25
0.73	7.1	19.4	40.7	154.2	−0.016	−0.082	−0.804	−4.440	4.55
0.79	7.0	19.3	39.7	179.1	−0.016	−0.088	−0.953	−4.400	4.73
0.86	7.0	19.2	38.8	179.6	−0.016	−0.096	−1.203	−4.360	4.69
0.89	6.9	19.2	38.5	185.9	−0.016	−0.100	−1.343	−4.340	4.61
0.95	6.8	19.0	37.7	186.9	−0.016	−0.105	−1.629	−4.320	4.36
1.06	6.7	18.8	36.3	181.0	−0.017	−0.119	−2.164	−4.310	3.71
1.19	6.4	18.5	34.8	186.5	−0.017	−0.135	−2.879	−4.370	2.78
1.36	6.2	18.2	36.1	190.5	−0.017	−0.159	−4.001	−4.570	1.93

temperature of the range tested ($T_{2-opt} = T_{ave} = 58.8$ °C), and the remained one at a temperature corresponding to 97% of the maximum extreme ($T_{3-opt} = 0.97T_{max} = 63{,}1$ °C). At each temperature, the sampling times correspond to 99.95% (T_{1-opt}), 97.49% (T_{2-opt}), 99.92% (T_{3-opt}) and 99.21% (T_{4-opt}) of inactivation (Fig. 2).

The heuristic experimental design used by Miller et al. (2009) (Table 2; considering all temperatures together) was compared to the *D*-optimal experimental design developed. The efficiency of the heuristic experimental design (ε) was calculated by the ratio between the determinant of the heuristic design (Δ_{heu}) and that of the *D*-optimal design (Δ_{opt}). Δ_{heu} was calculated considering the set of the six temperatures jointly, by substitution of the all 108 experimental times used by Miller et al. (2009); Δ_{opt} was calculated by substitution of T_{i-opt}/t_{i-opt}, $i = 1, \ldots 4$, each one replicated 24 times (*i.e.* 108/4), aiming at considering the same number of experimental points (Table 2). The values obtained were $\Delta_{heu} = 2.79 \times 10^{-1}$ and $\Delta_{opt} = 17.31$, which revealed an efficiency 1.6% of the heuristic design. If *D*-optimal design had been chosen, the confidence intervals at 95% of k_{ref}, E_a, A_{Ratk1} and A_{Ratk2} would decrease 64%, 88%, 80% and 72%, respectively, which shows that the application of *D*-optimal conditions would improve parameters estimation. The standard deviations of parameters estimates are in Table 4, for both heuristic and *D*-optimal designs.

4.2. Optimal design of experiments under time-varying temperature conditions

Non-isothermal conditions are a more complex situation compared with isothermal conditions, as not only the sampling times but also the heating rate affect parameter estimation. It was confirmed that, for a given heating rate (β), the sampling times affected the precision of the estimates and that the heating rate deeply influences the precision of the estimates. Fig. 3 shows an example of the variation of the determinant (Eq. (7)) with the heating rate, in a situation where the four sampling times, numerically calculated by maximization of the determinant, were the optimum ones for the heating rate and for the range of temperatures imposed (18.8–65.5 °C). The values are presented in Table 5, including the optimal sampling times and corresponding $\log(N/N_0)$. The existence of an optimal heating rate that leads to a maximum value of the determinant, and that consequently leads to maximum precision of the parameters estimates, is clear. The optimum heating rate was found to be 0.79 °C min^{-1}, which corresponds to the maximum of the absolute value of Δ obtained. Consequently, a *D*-optimal experiment for maximum parameter precision under non-isothermal conditions, using linear temperature histories from 18.8 to 65.0 °C, is the one with a heating rate of 0.79 °C min^{-1} and four samples taken at 7.0, 19.3, 39.7 and 179.1 min. These are feasible experimental conditions. The sampling times corresponds to 2.95%, 17.01%, 94.11% and 99.99% of microbial inactivation respectively.

However, if a non-optimum heating rate is chosen, it was found that the optimal sampling times were generally located in four periods of the inactivation kinetics: the first sample in the very beginning of the experiment, the second sample at the end of shoulder period, the third one when the maximum inactivation rate was reached and the last one when the asymptotic tail was attained. Curiously, t_{1-opt} corresponds approximately to 3.39–3.83% of inactivation and t_{4-opt} to 99.99%. Inactivation curves of *L. innocua* are in Fig. 4, assuming optimum (0.79 °C min^{-1}) and a non-optimum (2.50 °C min^{-1}) heating rates; optimal sampling times are represented for each situation. Please note that the last sampling point is not included in the figure, because it is an asymptotic value attained for a considerable high experimental time.

The efficiency of the experimental designs when heuristic samplings are chosen were calculated for the heating rates of 0.79 and 2.50 °C min^{-1}. In both cases a number of replicates were assumed, so that the two designs would have the same number of data points, for the sake of comparison (Table 6). For $\beta = 0.79$ °C min^{-1}, the ratio between Δ_{opt} calculated with 3 replicates of each optimal time (t_{1-opt}, t_{2-opt}, t_{3-opt} and t_{4-opt}) was compared to the one calculated with 12 sampling points equally spaced in a 120 min time scale (sampling in every 10 min). For $\beta = 2.50$ °C min^{-1}, the heuristic sampling was the one used by Gil (2009), assuming also 12 data points.

The efficiency of the heuristic design was 6.1% and 0.71%, for the heating rates of 0.79 °C min^{-1} and 2.50 °C min^{-1}, respectively (Table 6). It was further concluded that if *D*-optimal design was chosen, the confidence intervals at 95% of k_{ref}, E_a, A_{Ratk_1} and A_{Ratk_2} would decrease 1.7%, 225.1%, 82.5% and 30.5% respectively, if the optimum heating rate was considered. If the non-optimum heating rate was selected, the confidence intervals would decrease 2.0%, 26.5%, 134.4% and 176.9%. The standard deviations of parameters estimates are in Table 4, for both heuristic and *D*-optimal designs. These results show the importance of a careful selection of the heating rate for estimation of parameters under non-isothermal conditions.

Table 6
Heuristic sampling conditions and design efficiencies calculated on the basis of *D*-optimal design criterion for two non-isothermal conditions.

	β (°Cmin^{-1})			
	0.79		2.50	
	t_{heu} (min)	t_{opt} (min)	t_{heu} (min)	t_{opt} (min)
	10.0		0.5	
	20.0		2.0	
	30.0		4.0	
	40.0		6.0	
	50.0	4.1	8.0	4.1
	60.0	10.6	10.0	10.6
	70.0	17.9	12.0	17.9
	80.0	85.5	14.0	85.5
	90.0		16.0	
	100.0		18.0	
	110.0		20.0	
	120.0		22.0	
Δ_{heu}	2.36×10^{2}		3.76×10^{-1}	
Δ_{opt}		3.87×10^{3}		5.32×10^{1}
ε (%)	6.1		0.71	

5. Conclusions

If one is confident that a Gompertz-based model is adequate to describe the microbial thermal inactivation, the maximum inactivation rate and shoulder may be estimated with higher precision by performing one isothermal experiment where a number of replicates are taken at the times required to reach 89.09% and 99.97% of inactivation. If a range of temperatures is considered, four experiments should be conducted within the range considered: one at each extreme temperature, one at the average temperature of the range tested, and the remained one at a temperature corresponding to 97% of the maximum extreme ($T_{3-opt} = 0.97T_{max}$ °C). At each temperature, the sampling times (and replicates) correspond to 99.95% (at T_{1-opt}), 97.49% (at T_{2-opt}), 99.92% (at T_{3-opt}) and 99.21% (at T_{4-opt}) of inactivation.

If a non-isothermal methodology is applied, the optimal experimental design that leads to the maximum precision is more complex, as it also depends on the heating rate.

Although this work had focused on Gompertz-based kinetics, the *D*-optimal concept can be applied to any kinetic model, providing considerable benefits in parameter estimation.

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