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## Modelling combined effects of temperature and pH on the heat resistance of spores of *Bacillus cereus*

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1 Modelling combined effects of temperature and pH on the  
2 heat resistance of spores of *Bacillus cereus*.

3  
4 S. GAILLARD, I. LEGUERINEL, P. MAFART

5 Laboratoire Universitaire de Microbiologie Appliquée de Quimper  
6 Pôle Universitaire de Creach Gwen F29000 Quimper, France  
7

8 Abstract

9

10 The development of relationships between the pH of a heating medium and the  
11 thermal resistance of contaminant microorganisms is important and of a public  
12 health significance. A number of mathematical models have been presented in  
13 recent years, including that of Mafart and Leguérinel (1998) . However, in this  
14 This model , the effect of possible interactions between temperature and pH on  
15 D-values was not assessed. The consequences of ignoring interaction terms in  
16 models has been assessed and a comparison with Mafart's model that includes  
17 an interaction term showed that interaction terms can be neglected and that  
18 Mafart's model can be used in thermal process calculations. It appears possible  
19 to adopt a standard value of  $z_{pH}$  , for example the 3.6 value and the conventional  
20 concept of biological destruction value  $L(T)$  (ratio of the sterilization value and  
21 the exposure time at a fixed heating temperature) may then be extended to  
22  $L(T,pH)$  (the same ratio at a fixed temperature with a fixed pH of the heating  
23 menstruum).

24

25 Keywords:

26 Temperature, pH, Heat resistance, Spores, *Bacillus cereus*.

27

28

## 29 Introduction

30

31 Interactions between heating temperature and pH are frequently observed. In  
32 most cases, increasing temperature reduces the effect of pH. This type of  
33 interaction can be seen in the data of Xezones and Hutchings (1965) for  
34 *Clostridium botulinum*. This same behaviour is observed for *Bacillus*  
35 *stearothermophilus* (Rodrigo et al., 1993; Fernandez et al., 1994; Lopez et al.,  
36 1996). Rodrigo et al.(1993) observed that pH did not have any effect on the  $D_T$   
37 values of spores of *Clostridium sporogenes* when they were heated at high  
38 temperature. These results do not agree with those of Cameron et al.(1980) from  
39 which it can be shown that increasing temperature enhances the effect of pH.  
40 Acidifying the heating medium generally results in a decreased effect of  
41 temperature: an increase of the z value is observed when the pH of the medium  
42 decreases. This behaviour was showed for *Bacillus stearothermophilus*  
43 (Fernandez et al., 1994; Lopez et al., 1996), *Bacillus subtilis* (Condon and Sala,  
44 1992), *Bacillus licheniformis* (Palop et al., 1992) and *Clostridium sporogenes*  
45 (Silla Santos et al.1992). However, Cameron et al.(1980) pointed out an  
46 opposite effect for *Clostridium sporogenes*(a decrease of the z value with  
47 decreasing pH), that disagrees with results of Silla Santos et al.(1992) .

48 The mathematical relationship between the pH of the heating medium and  
49 the thermal resistance of microorganisms has been poorly documented. Jordan  
50 and Jacobs (1948) found a linear relationship between pH and the logarithm of  
51 the  $D_T$  values for *E. coli*. Davey et al.(1978) developed the first model for  
52 predicting the combined effects of both process temperature and pH on thermal  
53 resistance of bacteria. This empirical model , which was developed from data of  
54 Xezones and Hutching (1965) for inactivation of *Clostridium botulinum*, shows  
55 a satisfactory goodness of fit and is relatively parsimonious (4 parameters for 2  
56 factors). However it can be shown that Davey's model is still over  
57 parameterized (see appendix 1). The four empirical coefficients of the model  
58 have no biological significance. As an alternative, Mafart and Leguérinel (1998)  
59 proposed a new model with only three parameters, each having a  
60 physicochemical significance:

61

$$62 \log D_{(T,pH)} = \log D_{(T^*,pH^*)} - \frac{T - T^*}{z_T} - \frac{(pH - pH^*)^2}{z_{pH}^2} \quad (1)$$

63

64

65 Where  $T^*$  is the reference temperature (for example  $121.1^\circ\text{C}$ ),  $\text{pH}^*$  is the pH of  
66 maximal heat resistance of spores (generally pH 7),  $z_T$  is the conventional  
67 thermal z-value,  $z_{\text{pH}}$  is the distance of pH from  $\text{pH}^*$  which leads to a ten fold  
68 reduction of D value. Lastly,  $D_{(T^*, \text{pH}^*)}$  is the D value at  $T^*$  and  $\text{pH}^*$ .

69 This model was derived from the same data used by Davey et al., 1978 (i.  
70 e. Xezones and Hutching, 1965) and from two other sets of data related to  
71 *Clostridium sporogenes* (Cameron et al., 1980) and to *Bacillus*  
72 *stearothermophilus* (Lopez et al., 1996).

73 Neither Davey's nor Mafart's models took into account interactions  
74 between temperature and pH on thermal resistance of spores. This paper aims to  
75 check from our own data related to *Bacillus cereus* that the lack of term  
76 accounting for interactions in the Mafart's model can be justified.

77 The standard z-value used to compute most heating process is  $10^\circ\text{C}$   
78 because it nearly corresponds to the value for *Clostridium botulinum* spores and  
79 is closed to  $z_T$ -value of most other types of bacterial spores. Another aim of this  
80 paper is to check that the  $z_{\text{pH}}$  value of *Bacillus cereus* is in the same range than  
81 that of others studied types of spores. If this paper, confirmed by further works,  
82 shows that most of  $z_{\text{pH}}$  values are close to that of *Clostridium botulinum*, it  
83 would be possible to adopt a standard  $z_{\text{pH}}$  for calculations of heat treatments.

84

## 85 **Materials and methods**

### 86 **Microorganism and spore production**

87 The strain of *Bacillus cereus* (CNRZ 110) was obtained from the Institut  
88 National de Recherche Agronomie (France). Spores were kept in distilled water  
89 at  $4^\circ\text{C}$ .

90 Cells were precultivated at  $37^\circ\text{C}$  during 24 h in Brain Heart Infusion  
91 (Difco ). The preculture was used to inoculate nutritive agar plates (Biokar  
92 Diagnostics BK021) added with  $\text{MnSO}_4$   $40\text{mg l}^{-1}$  and  $\text{CaCl}_2$   $100\text{mg l}^{-1}$  on the  
93 surface aera. Plates were incubated at  $37^\circ\text{C}$  for 5 days. Spores were then  
94 collected by scraping the surface of the agar and suspended in sterile distilled  
95 water and washed three times by centrifugation ( $10000\times g$  for 15 min) (Bioblock  
96 Scientific, model Sigma 3K30). The pellet was then resuspended in 5 ml  
97 distilled water and 5 ml ethanol. The obtained suspension was then kept at  $4^\circ\text{C}$   
98 during 12 hours in order to eliminate vegetative non sporulated bacteria, and  
99 washed again three times by centrifugation. The final suspension (about  $10^{10}$   
100 spores  $\text{ml}^{-1}$ ) was at last distributed in sterile Eppendorfs microtubes and kept at  
101  $4^\circ\text{C}$ .

102

103 Thermal treatment of spore suspension

104  $D_{(T,pH)}$  values in citrate-phosphate buffers adjusted respectively to 4.1,  
105 4.5, 5.5, 6.5 and 6.9 were determined for temperatures of 86.6°C, 89°C, 95°C,  
106 101°C and 103.4°C with one replicate at each temperature and pH combination.  
107 The whole set of data involves a complete 3x3 factorial design from 89°C to  
108 101°C and from pH4.5 to pH 6.5 and in order to extend the range of validity of  
109 the model, the four following combinations were added: pH 5.5 at 86.6°C and  
110 103.4°C, pH 4.1 and 6.9 at 95°C.

111 First, 30µl of spore suspension was diluted in 3 ml buffer. Capillary tubes of 25  
112 µl (vitrex) were filled with 10µl of sample and submitted to a thermal treatment  
113 in a thermostated oil bath. After heating, the tubes were cooled in water/ice  
114 bath, washed in a solution of soap and rinsed with sterile distilled water.  
115 Finally, ends were flamed with ethanol. The capillary tubes were broken at both  
116 ends and their contents poured into a tube containing 9 ml sterile tryptone salt  
117 broth (Biokar Diagnostics) by rinsing with 1 ml tryptone salt broth contained in  
118 a syringe equipped with a needle.

119

120 Viable spore count

121 The viable spores were counted by duplicate plating in nutritive agar (10g  
122 tryptone, 5g meat extract, 5g sodium chloride, 15 g agar for 1000ml  
123 water)(Biokar Diagnostic) and incubating at 30°C for 48h.

124

125 Data analysis

126  $D_T$  values were based on the reciprocal of slopes obtained when the log  
127 number of survivors was plotted against time. Multiple linear regressions used  
128 to fit the model were carried out with the STAT-ITCF software (Institut  
129 Technique du Fourrage France).

130

131 The goodness of fit of the model was evaluated by using the per cent  
132 variance accounted for (Snedecor and Cochran, 1969) which is given by:

133

134 
$$R^2 = 1 - \frac{(1 - r^2)(n - 1)}{(n - N - 1)}$$

135

136 where n is the number of observations, N the number of terms and  $r^2$  is the  
137 multiple regression coefficient.

138

139

140

## 141 Results

142

143 Raw data of the experimental design are shown in Table 1. As the thermal  
 144 resistance of *Bacillus cereus* is relatively low, we adopted the standard  
 145 temperature  $T^*=100^\circ\text{C}$  instead of  $121.1^\circ\text{C}$ . On the other hand, a preliminary  
 146 experiment showed that the pH of maximal thermal resistance related to the  
 147 studied strain of *Bacillus cereus* was close to 7.5 (data not shown). The model  
 148 was then fitted with  $\text{pH}^*=7.5$ . Fitted parameters according to equation 1 were:

149

$$150 \quad D_{(T^*, \text{pH}^*)} = 60 \text{ seconds}$$

$$151 \quad z_T = 9.15^\circ\text{C}$$

$$152 \quad z_{\text{pH}} = 3.70$$

153 with a per cent variation accounted for  $R^2 = 97.7\%$ .

154 Families of graphs of  $D_{(T, \text{pH})}$  as a function of temperature at constant values of  
 155 pH and as a function of pH at constant values of temperature are shown in  
 156 Figures 1 and 2 respectively. Decreasing pH reduced the effect of temperature:  
 157 at pH 6.5, the  $z_T$  value was  $7.97^\circ\text{C}$  whilst it becomes  $9.27^\circ\text{C}$  and  $10.3^\circ\text{C}$  at  
 158 pH 5.5 and 4.5 respectively. At  $95^\circ\text{C}$  and  $101^\circ\text{C}$ , the  $z_{\text{pH}}$  value remains constant  
 159 and equal to 3.87. On the other hand, a decrease of the  $z_{\text{pH}}$  value is observed at  
 160  $89^\circ\text{C}$  ( $z_{\text{pH}}=3.06$ ). It is then clear that the heat resistance of spores depends not  
 161 only on temperature and pH of the medium, but also on interactions between  
 162 these two factors. In order to take into account interactions, the model (1) was  
 163 modified by adding an interaction term :

164

$$165 \quad \log D_{(T, \text{pH})} = \log D_{(T^*, \text{pH}^*)} + C_1(T - T^*) + C_2(\text{pH} - \text{pH}^*)^2 + C_3(T - T^*)(\text{pH} - \text{pH}^*)^2$$

166

$$167 \quad (2)$$

168 Where  $C_1$ ,  $C_2$  and  $C_3$  are empirical parameters without known physiological  
 169 significance. Fitted parameters according to this new model were:

$$170 \quad D_{(T^*, \text{pH}^*)} = 83 \text{ seconds}$$

$$171 \quad C_1 = -0.2900^\circ\text{C}^{-1}$$

$$172 \quad C_2 = -0.0281$$

$$173 \quad C_3 = 0.1807^\circ\text{C}^{-1}$$

174 with a per cent variation accounted for  $R^2 = 98.5\%$ .

175

176 The new interaction term was highly significant ( $P < 0.01\%$ )

177 The comparison between goodness of fit of both models (equations 1 and 2)  
178 are illustrated by Figures 3 and 4.

179

#### 180 4. Discussion

181

182 Variation of the thermal resistance of *Bacillus cereus* spores as a function of  
183 temperature and pH is similar to those for many bacterial spores. It is confirmed  
184 that the pH of the heating medium has a prominent effect on the  $D_{(T,pH)}$  values.

185 For example, at 95°C a decrease of D-value of 5.7 times can be observed when  
186 the pH decrease from 6.9 to 4.1. As for of most bacterial spores, an increase of  
187 the temperature results in a decrease in the effect of the pH while a decrease of  
188 the pH medium results in a decrease of the effect of the temperature. This  
189 behaviour means that some interactions on  $D_{(T,pH)}$ -values exist between  
190 temperature and pH. This is confirmed by the fact that the interactions term of  
191 equation (2) is highly significant. However, adding one more parameters to our  
192 model in order to account for interactions results in a poor improvement of its  
193 goodness of fit : (with a R<sup>2</sup>-value of 0.985 instead of 0.977). Moreover, in  
194 Equation 2, only one parameter ( $D_{(T^*,pH^*)}$ ) keeps a biological significance:  
195 because of the occurrence of the interaction term, it is not possible to  
196 reparameterize C<sub>1</sub> and C<sub>2</sub> into z<sub>T</sub> and z<sub>pH</sub> respectively. Fernandez et al.(1996)  
197 proposed two models for combined effects of temperature and pH on the  
198 thermal resistance of *Bacillus stearothermophilus* and *Clostridium sporogenes*:  
199 one a polynomial quadratic model including an interaction term (6 parameters),  
200 the other a simple linear model without interaction terms (3 parameters). From  
201 their data it may be concluded that the slight improvement of goodness of fit  
202 obtained by the quadratic model with respect to the linear model does not justify  
203 the choice of the 6 parameters model rather than that of 3 parameters model.  
204 The linear model of Fernandez et al. is of the form:

$$205 \quad \log K = a + bT + cpH \quad (3)$$

206 where K is the death rate of spores and a, b and c empirical coefficients. It  
207 can be easily shown (see Appendix 2) that equation(3) can be reparameterized to  
208 give:

$$209 \quad \log D = \log D^* - \frac{1}{z_T} (T - T^*) - \frac{1}{z_{pH}} (pH - pH^*) \quad (4)$$

210

211 Disregarding pH terms, both models (1) and (4) are reduced to Bigelow's  
212 equation. However equation (1) where the pH term is squared differs from the



213 simple linear model (equation (4)) and is the only one to take the sigmoidal  
214 pattern of D values curves versus pH into account.

215 The  $z_{pH}$  value (3.7) related to *Bacillus cereus* have the same magnitude  
216 that the observed values for *Clostridium botulinum* (3.56-3.61), *Clostridium*  
217 *sporogenes* (3.33-4.29) and *Bacillus stearothermophilus* (2.94-3.97) (Mafart  
218 and Leguérinel, 1998). Like conventional  $z_T$  values of spores close to 10°C and  
219 keep generally the range of 8°C to 12°C, it appears that  $z_{pH}$  values are, in most  
220 situations, included in a 3 to 4 range. If this observation is confirmed, it may be  
221 possible to adopt a standard value of  $z_{pH}$  (for example the 3.6 value of  
222 *Clostridium botulinum* ) in order to include the pH in thermal process  
223 calculations. The sterilization value could be defined as a heat treatment  
224 equivalent to an exposure time of 1 min at 121.1°C and at pH 7. The  
225 conventional concept of biological destruction value L(T) could then be  
226 extended to L(T,pH) with

$$227 \quad L(T, pH) = 10^{\frac{T - T^*}{z_T} + \left( \frac{pH - pH^*}{z_{pH}} \right)^2} \quad (5)$$

228  
229 This function corresponds to the exposure time, at a T temperature and at a  
230 given pH, which would be necessary in order to obtain one sterilization unit.

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constituents. *Food Technol.* **19**, 1003-1005.

286 Appendix 1: Reparameterization of Davey's model

287 Davey et al.(1978) described variation of death rates versus temperature  
288 and pH by the following equation:

$$289 \quad \text{Ln}K = C_0 + \frac{C_1}{T} + C_2 \text{pH} + C_3 \text{pH}^2 \quad (1)$$

290 where K is the death rate constant, T is the absolute temperature and C<sub>0</sub>,  
291 C<sub>1</sub>, C<sub>2</sub> and C<sub>3</sub> are empirical coefficients. Let pH\* be the pH value of maximal  
292 thermal resistance of spores (in most cases pH\*=7)

293 In the situation of maximal thermal resistance,

$$294 \quad \frac{d(\text{Ln}K)}{d\text{pH}} = C_2 + 2C_3 \text{pH} = 0 \quad (2)$$

295

$$296 \quad \text{pH}^* = \frac{-C_2}{2C_3} \quad (3)$$

297 so that Davey's model can be rewritten with only three parameters:

$$298 \quad \text{Ln}K = C_0 + \frac{C_1}{T} + C_2 \text{pH} \left( 1 - \frac{1}{2} \frac{\text{pH}}{\text{pH}^*} \right) \quad (4)$$

299 For example, in the case of the heat treatment of *Clostridium botulinum*  
300 spores in spaghetti tomato sauce, Davey's coefficient values were C<sub>2</sub>=-2.3967;  
301 C<sub>3</sub>=0.1965. In macaroni creole, C<sub>2</sub>=- 2.6170 and C<sub>3</sub>= 0.1871. Corresponding  
302 pH\* values are then 7.07 and 6.99 respectively.

303

304 Appendix 2: Reparameterization of Fernandez's linear model

305 The following model was proposed:

306  $\log K = a + bT + cpH$  (1)

307 with  $K = \frac{Ln10}{D}$

308 so equation (1) is equivalent to

309  $\log D = \log(Ln10) - a - bT - cpH$  (2)

310 or

311  $\log D = \log D^* - \frac{1}{z_T}(T - T^*) - \frac{1}{z_{pH}}(pH - pH^*)$  (3)

312 By comparison between equation (2) and (3) new parameters  $D^*$ ,  $z_T$  and  
313  $z_{pH}$  can be identified versus Fernandez's parameters:

314  $\log D^* = \log(Ln10) - a - bT^* - cpH^*$

315  $z_T = \frac{1}{b}$  (5)

316  $z_{pH} = \frac{-1}{c}$  (6)

317 For example, for *Bacillus stearothermophilus* heated in mushroom extract  
318 acidified with glucono- $\delta$ -lactone,  $a=-12.0495$ ;  $b=0.1156$ ;  $c=0.2990$ . With  
319  $T^*=121.1^\circ\text{C}$  and  $pH^*=7$ , new parameters are

320  $D^*=3.20$  minutes

321  $z_T=8.65^\circ\text{C}$

322  $z_{pH}=3.34$

323

324

325

326

Table 1

327

pH/T	86.6°C	89°C	95°C	101°C	103.4°C
4.1			43.2 46.5		
4.5		158.2 156.4	51.9 45.4	10.9 10.6	
5.5	706 692	394.8 411.0	92.1 92.0	21.8 21.4	10.6 10.1
6.5		1185 1189	180.8 197.8	38.2 36.1	
6.9			257.3 256.6		

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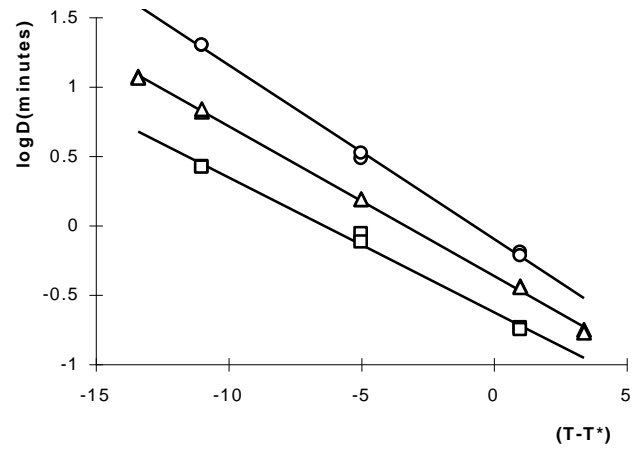
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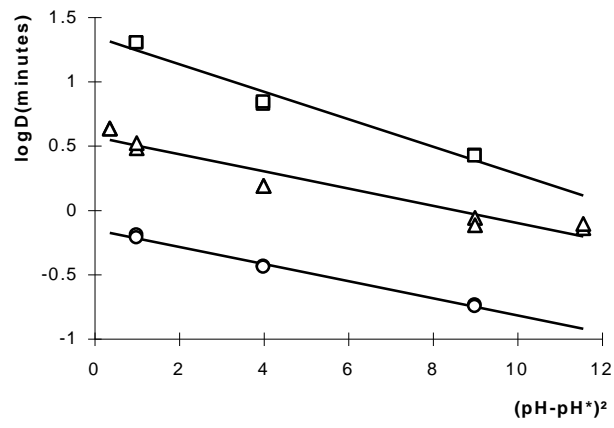


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figure 1

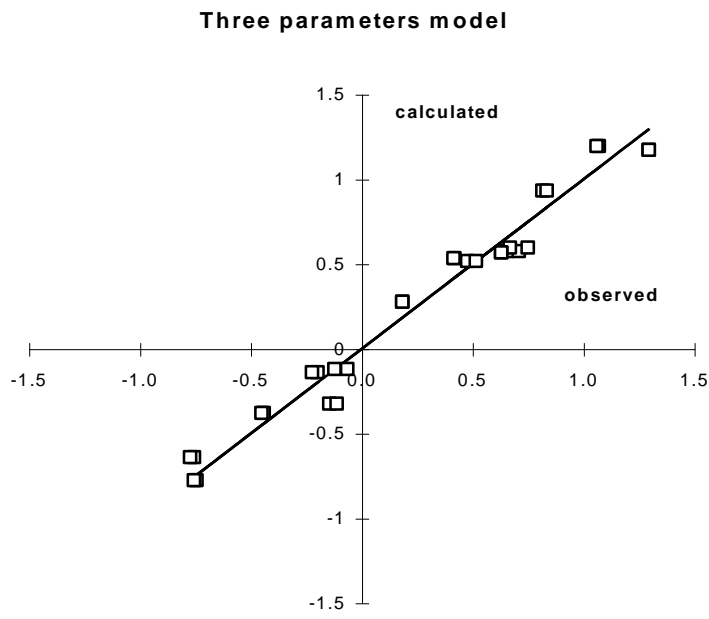


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figure2



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Figure 3

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**Erreur! Objet incorporé incorrect.**

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362

363

364 Figure 4

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367

368 Legends of figures

369

370 Figure 1 :

371 Log D value of *Bacillus cereus* spores versus (T-T\*).

372 Key : □, log D at pH 6.5 ; Δ, log D at pH 5.5 ; ○, log D at 4.5.

373

374 Figure 2 :

375 Log D value of *Bacillus cereus* spores versus (pH-pH\*)

376 Key : □, log D at 89°C ; Δ, log D at 95°C ; ○, log D at 101°C.

377

378 Figure 3 :

379 Observed log D value compared to log D value calculated according to the three  
380 parameters model

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382 Figure 4 :

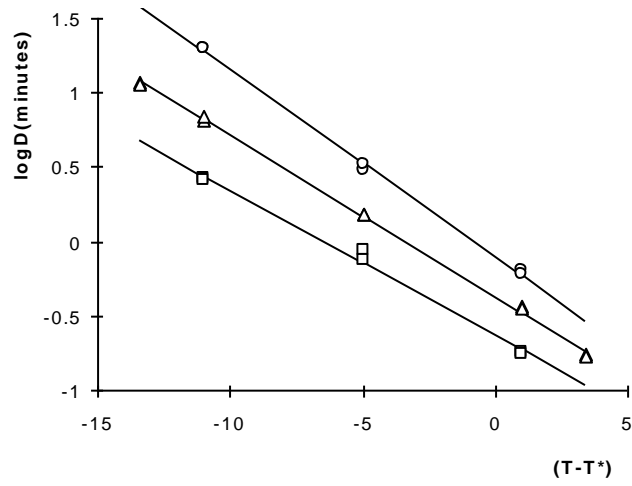
383 Observed log D value compared to log D value calculated according to the four  
384 parameters model

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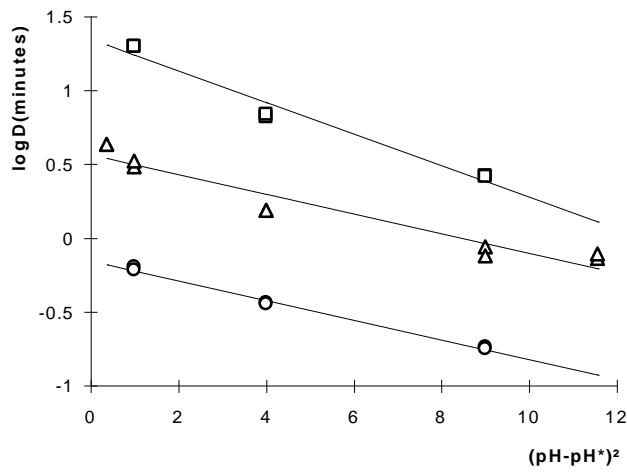
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387 Legends of Table  
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389 Table 1  
390 D values (minutes) of experimental design.  
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