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1 Effect of the water activities of the heating and the recovery media on  
2 the apparent heat resistance of *Bacillus cereus* spores.

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10

11 **Spores of *Bacillus cereus* were heated and recovered in order to investigate the effect of water activity of**  
12 **media on the estimated heat resistance (D-value) of spores. The water activity (ranging from 0.9 to 1) of**  
13 **the heating medium was first successively controlled with three solutes (glycerol, glucose and sucrose)**  
14 **while the water activity of the recovery medium was kept near 1. Reciprocally, the water activity of the**  
15 **heating medium was then kept to 1, while water activity of the recovery medium was controlled from 0.9**  
16 **to 1 with the same depressors. Lastly, in a third set of experiments, the heating medium and the recovery**  
17 **medium were adjusted to the same activity. As expected, added depressors caused an increase of the heat**  
18 **resistance of spores with a greater efficiency of sucrose with respect to glycerol and glucose. On the**  
19 **contrary, when solutes were added to the recovery medium, under an optimal water activity closed to**  
20 **0.98, a decrease of water activity caused a decrease of estimated D-values. This effect was more**  
21 **pronounced when sucrose was used as a depressor instead of glycerol or glucose. When the heating and**  
22 **the recovery media were adjusted to the same water activity, a balancing effect was observed between the**  
23 **protective influence of solutes during heat treatment and their negative effect during the recovery of**  
24 **injured cells, so that the overall effect of water activity was reduced, with an optimal value near 0.96. The**  
25 **difference between the efficiency of depressors was also less pronounced. It may then be concluded that**  
26 **the overall protective effect of a decrease in water activity is generally overestimated.**

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28  
29 It has been recognized that the heat resistance of bacterial spores depends on the medium in  
30 which spores are heated. The maximum thermostability of most microorganisms was found in  
31 the range between 0.2-0.4 water activity (1, 3, 27, 28, 30). In typical ranges of water activities  
32 which are found in foodstuffs ( $a_w > 0.8$ ), the heat resistance of microorganisms generally

33 increases at decreasing water activities. However, the apparent effect of water activity of the  
34 medium on spores or vegetative cells is complicated by the specific effect of solutes which are  
35 used as depressors. It is generally agreed that the occurrence of such solutes in the medium  
36 reduces the heat resistance of microorganisms. This antagonism between the protective effect  
37 of an increase in water activity and the opposite specific effect of depressors can explain  
38 conflicting data from various authors.

39 The influence of salt on the thermostability of microorganisms is disputed and depends on the  
40 heated type of microorganism. Some authors found no effect of the sodium chloride  
41 concentration on the heat resistance of bacteria (9, 29, 32, 42). Others observed a reduced heat  
42 resistance of microorganisms at increasing salt concentration (7, 12, 22, 23). On the contrary,  
43 a protective effect of salt was found by several authors (6, 14, 26, 35, 38, 39, 40). Corry (14)  
44 deduced from his data that sodium chloride had a thermal protective effect on most heat  
45 sensitive bacteria and the opposite effect on most heat resistant species. Other solutes show  
46 the same opposite influence between their common depressor character which protects spores  
47 against heat and their specific effect which, on the contrary, reduces their heat resistance. It  
48 was observed (21) that an increase of the thermal resistance of spores was more pronounced  
49 when the decrease of the medium water activity was generated by drying instead of an  
50 addition of glycerol, sodium chloride, lithium chloride or glucose. Baird- Parker et al., (5)  
51 could not find any correlation between D-values of Salmonellae and the water activity of  
52 heating media when sodium chloride or glycerol were used as depressors. However, they  
53 observed a clear protective effect of sucrose, more pronounced for most heat sensitive strains.  
54 It is generally recognized that sucrose is the most protective depressor while glucose, sodium  
55 chloride and lithium chloride show a clearly lower influence or even an opposite effect.  
56 Glycerol shows an intermediate behavior (13, 19, 20, 26, 37). Interactions between influences  
57 of water activity and of heating temperature were often observed. An increase of D-values

58 generated by a reduced water activity of the heating medium is generally related to an increase  
59 of z-values. Moreover, several workers demonstrated that the effect of the water activity of the  
60 heating medium depended on the treatment temperature: for example, in the case of  
61 *Staphylococcus epidermidis* (39) or *Listeria monocytogenes* (37) the protective effect of  
62 decreasing water activity is more pronounced at higher treatment temperature while the  
63 opposite trend was observed for *Staphylococcus aureus* (38). A few predictive models  
64 describing the effect of the water activity of the heating medium on the heat resistance of  
65 spores were developed (8, 18, 31).

66 The nature of the recovery medium in which surviving heated cells are incubated have a great  
67 influence on their apparent heat resistance, i.e. their estimated D-value (24). It is generally  
68 agreed that there is an optimum temperature of incubation for the cell ratio of recovery (16,  
69 36) and the apparent D-value (10). Acidification of the recovery medium causes also a  
70 reduction in spore recovery and in apparent heat resistance (11, 17, 33, 34, 41). Addition of  
71 sodium chloride in the recovery medium causes effects similar to those observed with  
72 acidification: a reduction of the viability of cells and a lower apparent D-value (7, 12, 22, 29,  
73 32). However, as far as we know, the effect of reducing the water activity of the recovery  
74 medium by other depressors than sodium chloride had never been investigated.

75 The purpose of this work was to investigate and to describe from a predictive model the  
76 influence of the water activity of the recovery medium with glycerol, glucose and sucrose used  
77 as depressors, upon the apparent D-value of *Bacillus cereus* spores.

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## MATERIALS AND METHODS

81

82     **Microorganism and spore production.** The strain of *Bacillus cereus* (CNRZ 110) was  
83 obtained from the Institut National de Recherche Agronomique (France). Spores were kept in  
84 distilled water at 4°C. Cells were precultivated at 37°C during 24 hrs in Brain Heart Infusion  
85 (Difco ). The preculture was used to inoculate nutritive agar plates (Biokar Diagnostics  
86 BK021) added with  $\text{MnSO}_4$  40mg l<sup>-1</sup> and  $\text{CaCl}_2$  100 mg l<sup>-1</sup> on the surface area. Plates were  
87 incubated at 37°C for 5 days. Spores were then collected by scraping the surface of the agar  
88 and suspended in sterile distilled water and washed three times by centrifugation (10000xg for  
89 15 min) (Bioblock Scientific, model Sigma 3K30). The pellet was then resuspended in 5 ml  
90 distilled water and 5 ml ethanol. The obtained suspension was then kept at 4°C during 12  
91 hours in order to eliminate vegetative non sporulated bacteria, and washed again three times  
92 by centrifugation.

93 Lastly the final suspension (about 10<sup>10</sup> spores ml<sup>-1</sup>) was at last distributed in sterile Eppendorfs  
94 microtubes and kept at 4°C.

95     **Thermal treatment of spore suspension.** D values in citrate-phosphate buffers adjusted  
96 were determined at 95°C with one replicate at each a<sub>w</sub> value ranging from 1 to 0.89.  
97 Three solutes (glycerol, glucose, sucrose) were used to adjust the water activity value. The  
98 previous molarities of the different solutes were determined using curves from model  
99 UNIFAC-LARSEN (2). The heating medium was sterilized by filtration and the a<sub>w</sub> values  
100 were controlled with an a<sub>w</sub>-meter ( FA-st1 GBX France Scientific Instrument).

101 First, 30µl of spore suspension was diluted in 3 ml heating medium. Capillary tubes of 25 µl  
102 (vitrex) were filled with 10µl of sample and submitted to a thermal treatment in a  
103 thermostated oil bath. After heating, the tubes were cooled in water/ice bath, washed in a  
104 solution of soap and rinsed with sterile distilled water. Finally, ends were flamed with ethanol.  
105 The capillary tubes were broken at both ends and their contents poured into a tube containing

106 9 ml sterile tryptone salt broth (Biokar Diagnostics) by rinsing with 1 ml tryptone salt broth  
107 contained in a needle-equipped syringe.

108 **Recovery conditions.** Viable spores were counted by duplicate plating at different  $a_w$  values  
109 in nutritive agar (10g tryptone, 5g meat extract, 5g sodium chloride, 15 g agar for  
110 1000ml)(Biokar Diagnostic) and incubated at 25°C for 6 to 21 days. The  $a_w$  ranging from 1 to  
111 0.92 was adjusted with glycerol, glucose or sucrose. To adjust  $a_w$  values, the previous  
112 molarities of the different solutes were determined using curves from model UNIFAC-  
113 LARSEN (2). Nutritive agar was sterilized by autoclaving, glycerol, glucose or sucrose  
114 solution were sterilized by filtration to avoid Maillard reaction. After sterilization the two  
115 solutions were mixed, the pH was adjusted to 7 and the  $a_w$  value was controlled.

116 **Data analysis.** D values were determined on the straight portion of curves obtained when  
117 the log number of survivors was plotted against time.

118 The parameters of the models were estimated by simple linear regression carried out with  
119 MINITAB software. The goodness of fit of the model was evaluated by using the per cent  
120 variance  $R^2$ .

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122

## RESULTS

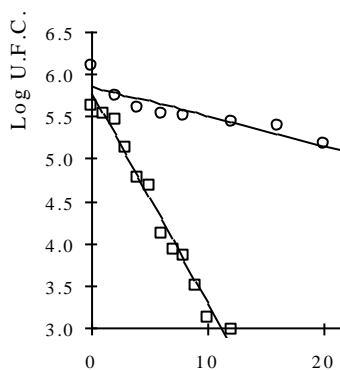
123

124 **Effect of the water activity of the heating medium.** For identical heat treatment, the  
125 recovery conditions influence the apparent heat resistance of bacterial spores (fig. 3 & 4). A  
126 clear protective effect on spores of *B. cereus* heated at 95°C and at pH 7 was observed when  
127 solutes were added to the heating medium for the three types of depressors (Fig.1 & 2).  
128 However, it can be seen that the effect of sucrose is more pronounced than that of glycerol and  
129 glucose. While a  $D_{95^\circ\text{C}}$  of about 4 min at water activity closed to 1 was found, at water activity

130 0.9, observed  $D_{95^{\circ}\text{C}}$  became closed to 11.2 min, 10.5min and 27 min for glycerol, glucose and  
131 sucrose respectively.

132 The three sets of data corresponding to each depressor were fitted according to the Gaillard et  
133 al. model (18) which, at isothermal conditions and at a fixed pH of the heating medium can be  
134 reduced to:

135



136 Where  $D_{(a_w,1)}$  is the estimated D-value at a water activity of the heating medium  $a_w$  and a  
137 water activity of the recovery medium of 1.  $z^*_{a_w}$  corresponds to the decrease in water activity  
138 of the treatment medium which would cause a tenfold reduction of the decimal reduction  
139 time, with a water activity of the recovery medium of 1. Estimated parameter values are  
140 presented in Table 1.

141 **Effect of the water activity of the recovery medium.** Whatever the solute used as  
142 depressor in the recovery medium, heated spores show the same maximum apparent heat  
143 resistance ( $D_{95^{\circ}\text{C}}$ -value of about 5 min), at an optimum water activity closed to 0.98 (see Fig.  
144 2). Under this optimal value, an increasing concentration of the three depressors causes a  
145 decrease in the apparent heat resistance of spores. Sucrose presents the most pronounced  
146 effect, followed in turn by glucose and glycerol. At water activity 0.92, estimated  $D_{95^{\circ}\text{C}}$  values  
147 were 0.9 min, 1.9 min and 2.5 min with sucrose, glucose and glycerol respectively.

148 We tried to adapt the model describing the influence of the pH of the recovery medium on the  
149 apparent thermal resistance of spores, which was developed in our laboratory (15) by  
150 substituting pH for water activity, which leads to the following equation:

151

152

153 Where  $D'_{(1,a'w)}$  is the estimated D-value at a water activity of the recovery medium  $a'_w$  and a  
154 water activity of the heating medium of 1.  $z'_{aw}$  corresponds to the decrease of water activity  
155 of the recovery medium which would cause a tenfold reduction of the decimal reduction time,  
156 with a water activity of the heating medium of 1. Estimated parameter values are presented in  
157 Table 2.

158 **Overall effect of water activity of foods on the apparent heat resistance of spores.** As  
159 foods make up both the heating medium and the recovery medium, a third set of experiments  
160 was carried out, in which spores were recovered at the same water activities as those of the  
161 heating menstroom.(Fig. 3). With respect to the second set of data in which the water activity  
162 of the heating medium was kept to 1, some noteworthy differences appear. First, the overall  
163 influence of water activity becomes relatively slight, while differences of curves patterns  
164 according to used depressors are less pronounced than those of Fig. 2 .

165 Secondly, a shift of the optimum water activity from 0.98 toward 0.96 can be observed, with  
166 a maximum  $D_{95^\circ C}$  value closed to 8 min instead of 5 min. Equations 1 and 2 cannot directly be  
167 combined in order to build a model which would take into account the overall effect of the  
168 food water activity because they were developed by keeping the water activity of the recovery  
169 medium to 1 for equation 1, and the water activity of the heating medium to 1 for equation 2.  
170 The accuracy of this third set of data was too poor to allow an attempt of suitable modeling.



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173

## DISCUSSION

174

175 It is confirmed that an increase of the water activity produces opposite effects on the apparent

176 heat resistance of spores according whether it concerns the heat treatment either the recovery

177 medium. Inside the investigated water activity range (0.9-1) which corresponds to that of

178 most typical foods, a clear protective effect of an increasing water activity of the heating

179 medium can be observed. For a fixed water activity, the degree of protection depends on the

180 type of used depressor: according to our investigations, sucrose showed a more effective

181 protective effect than glycerol and glucose, which is in agreement with observations by other

182 authors (5, 19, 26, 37). Like in the case of NaCl, the protective influence of an increase of the

183 water activity could partly be balanced by a specific antagonistic and toxic effect of glycerol

184 and glucose . Moreover, the plasmolyse which is partly responsible for the heat protection of

185 spores is limited by the penetration of glycerol and glucose inside cells. This limitation does

186 not exist when the depressor is not uptaken inside cells, which is the case of sucrose. Another

187 explanation for the protective effect of a depressor in the heating medium could be an

188 inhibition of spore germination. Anagnostopoulos and Sidhu (4) observed for *Bacillus*

189 *stearothermophilus* that the percentage of germination decreases when the water activity

190 decreased and that, at the same water activity, the percentage of germination was lower in a

191 nutrient broth supplemented with sucrose than in a broth supplemented with glycerol.

192 Hydration of spore protoplast is an important condition of spore activation and initiation of

193 germination. Germination is inhibited in the absence of moisture or in concentrated solution

194 of non penetrating solute. The spores which do not germinate are protected during heat

195 treatment and can germinate and growth during recovery. This explanation is in agreement

196 with our results : when the water activity decreases, the heat resistance of spores increases,  
197 and , at the same water activity, sucrose, a less penetrating solute in protoplast than glycerol  
198 and glucose, shows a more protective effect than these two solutes. Moreover, according to of  
199 Anagnostopoulos and Sidhu's data, the  $z_{aw}$  values for glycerol and sucrose (0.28 and 0.13  
200 respectively) correspond to the  $a_w$  value difference which leads to a tenfold reduction of the  
201 percentage of germination of *Bacillus stearothermophilus* at 75°C with glycerol and sucrose  
202 (0.31 and 0.11 respectively).

203

204 It is recognized that addition of sodium chloride in the recovery medium causes both a  
205 reduction of viability of cells and a lower apparent D-value of spores (7, 12, 16, 22, 32).  
206 However, as far as we know, the influence of the water activity of the recovery medium and  
207 types of used depressors upon estimated D-values of spores, had never been investigated. In  
208 our experimental conditions, a maximal apparent heat resistance of spores appeared at an  
209 optimal water activity closed to 0.98. Under this value, a decrease in the water activity of the  
210 recovery medium causes a reduction of the estimated D-value of spores. It is interesting to  
211 note that towards this trend, depressors appear at the same increasing order of effectiveness  
212 than that which was found regarding their protective effect in the heating medium: glycerol,  
213 glucose and sucrose respectively. This order corresponds also to the increasing order of  
214 molecular weights and to the decreasing order of degree of penetration inside cells.  
215 Particularly, the absence of uptake of sucrose by cells keeps a sharp gradient of osmotic  
216 pressure between the cell inside and the outside medium, which reduces the viability of  
217 surviving cells.

218 The overall influence of water activity of a single medium which makes up both the heating  
219 menstruum and the recovery medium upon apparent D-values of spores had never been  
220 investigated. Our results show that the protective effect of a decrease in water activity of the

221 medium during heat treatment is more or less offset by a reduction of the viability of surviving  
222 cells during the recovery. Moreover, because depressors, which are the most effective  
223 regarding the heat protection of spores, are also responsible for the maximum loss of viability  
224 of injured cells, their overall difference of efficiency is greatly reduced. While most authors  
225 locate the optimal water activity of maximum heat resistance of spores between 0.2 and 0.4,  
226 because they recovered their surviving cells at optimal conditions, when media of heat  
227 treatment and of incubation make up a same and single medium, like for heat processed foods,  
228 the optimal water activity is actually near 0.96.

229 Investigating separately the influence of water activity of the heating medium on the heat  
230 resistance of spores on the one hand, and the effect of water activity of the recovery medium  
231 on the viability of surviving cells on the other hand, obviously provides very interesting and  
232 useful data: these two effects must be regarded as two different factors which interact with  
233 each other. However, when the heating medium also makes up the recovery medium, it is  
234 worth to investigating the overall influence of water activity on the apparent heat resistance of  
235 spores, which reflects both its immediate thermal resistance during heating and its ability to  
236 grow in a recovery medium.

237 In the framework of predictive microbiology, we could develop two separate models for  
238 describing the effect of water activity of the heating menstruum on the one hand, and that of  
239 water activity of the recovery medium on the other hand, upon the apparent heat resistance of  
240 spores. However, further works would be needed in order to develop an overall predictive  
241 model adapted for heat processed food calculations. Such a model would take into account  
242 both opposite effects of water activity and would allow to improve of heat treatment  
243 optimization.

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Table 1

| Depressor   | Glycerol | Glucose  | Sucrose  |
|-------------|----------|----------|----------|
| $D_{(1,1)}$ | 4.81 min | 4.54 min | 3.36 min |
| $z^*_{aw}$  | 0.284    | 0.274    | 0.129    |
| $R^2$       | 0.921    | 0.872    | 0.921    |

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Table 2

| Depressor   | Glycerol | Glucose  | Sucrose  |
|-------------|----------|----------|----------|
| $D_{(1,1)}$ | 4.75 min | 4.55 min | 4.52 min |
| $z'_{aw}$ * | 0.103    | 0.113    | 0.068    |
| $R^2$       | 0.893    | 0.883    | 0.941    |

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Legend of figures

Figure 1

Log UFC vs. heating time for *Bacillus cereus* CNRZ 110 heated at 95°C, pH7, aw 1 (□) or 0.9 (○) adjusted with sucrose, the aw value of the recovery condition equal 1.

Figure 2a

*Bacillus cereus* D<sub>95°C</sub> value vs aw of the heated medium adjusted with glycerol : ○ experimental data, — calculated values

Figure 2b

*Bacillus cereus* D<sub>95°C</sub> value vs aw of the heated medium adjusted with glucose : □ experimental data, — calculated values

Figure 2c

*Bacillus cereus* D<sub>95°C</sub> value vs aw of the heated medium adjusted with sucrose : Δ experimental data, — calculated values

Figure 3

Log UFC vs. heating time for *Bacillus cereus* CNRZ 110 heated at 95°C, pH7, aw 1 incubated at 25°C in recovery medium at aw 1 ■ or aw 0.92 ● adjusted with sucrose.

Figure 4a

*Bacillus cereus* D<sub>95°C</sub> value vs aw of the recovery medium adjusted with glycerol : ● experimental data, — calculated values

Figure 4b

*Bacillus cereus* D<sub>95°C</sub> value vs aw of the recovery medium adjusted with glucose : ■ experimental data, — calculated values

Figure 4c

*Bacillus cereus* D<sub>95°C</sub> value vs aw of the recovery medium adjusted with sucrose : ♦ experimental data, — calculated values

Figure 5a

*Bacillus cereus* D<sub>95°C</sub> value vs aw of both heated and recovery medium adjusted with glycerol : ● experimental data, — calculated values

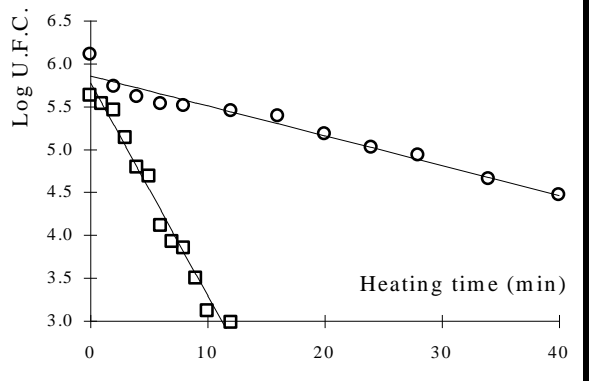
Figure 5b

*Bacillus cereus* D<sub>95°C</sub> value vs aw of both heated and recovered medium adjusted with glucose : ■ experimental data, — calculated values

Figure 5c

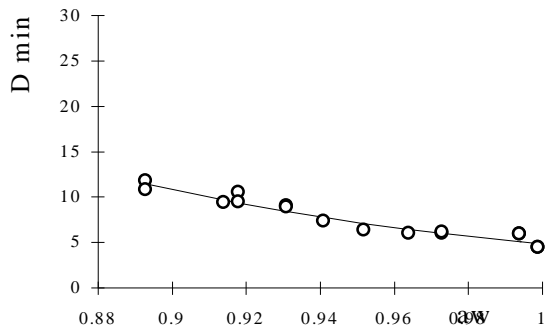
*Bacillus cereus* D<sub>95°C</sub> value vs aw of both heated and recovery medium adjusted with sucrose : ♦ experimental data, — calculated values

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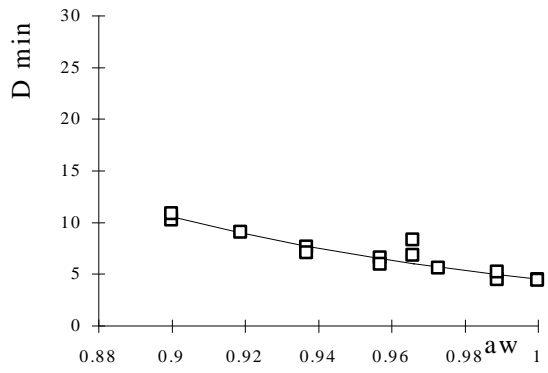


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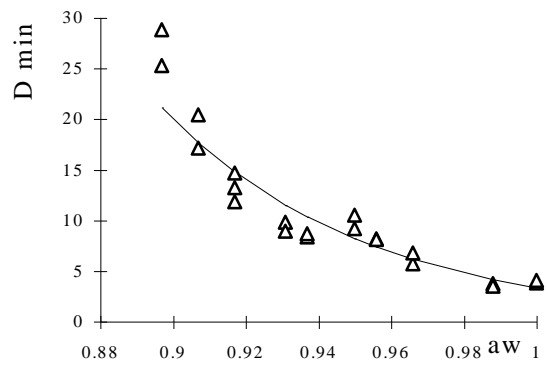
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438 Figure 2a  
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441 Figure 2b  
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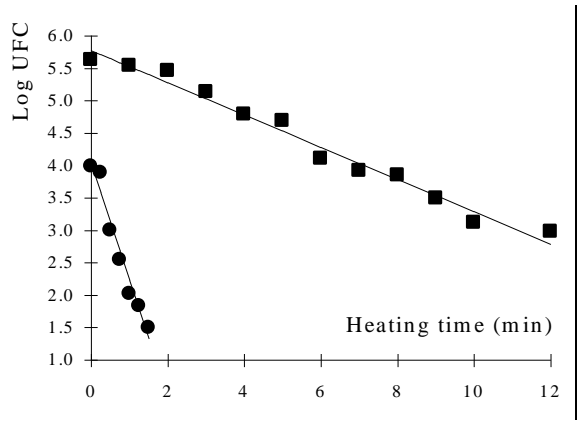


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444 Figure 2 c  
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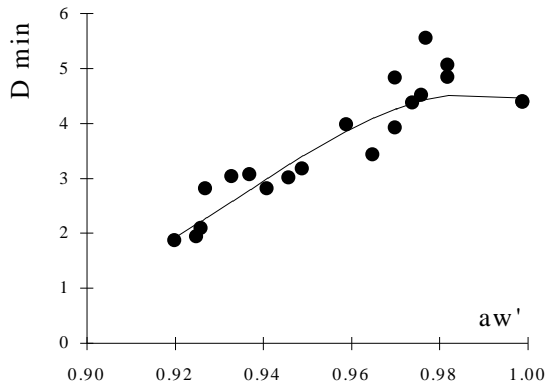
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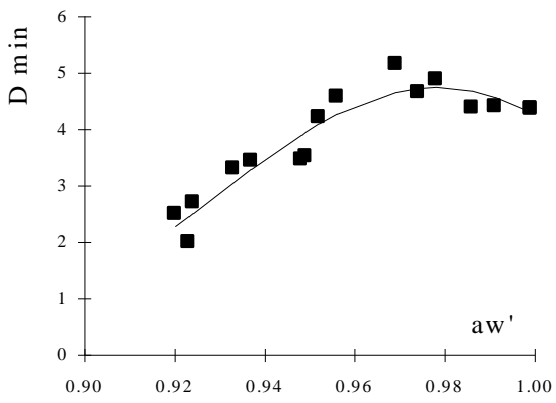


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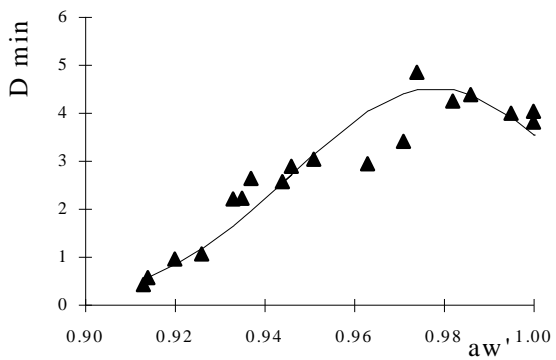
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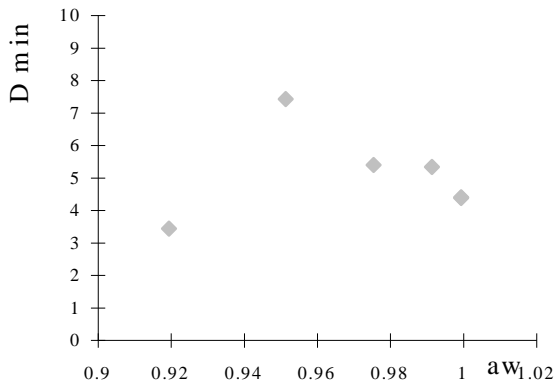


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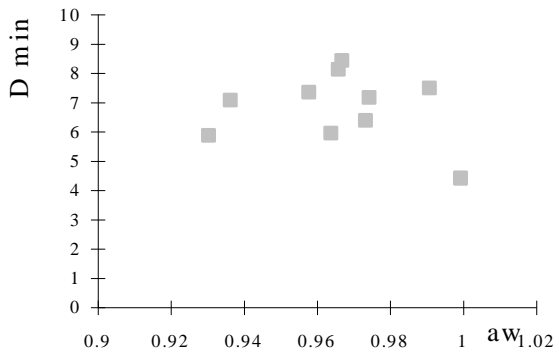


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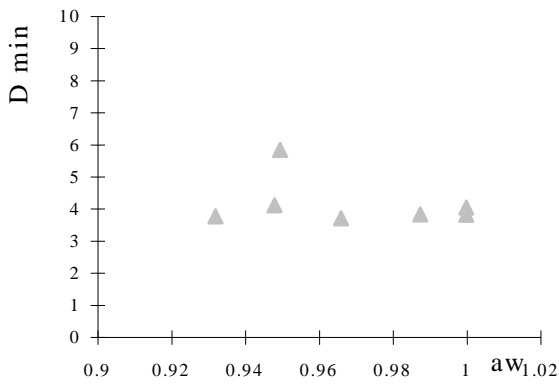
467  
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