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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Microorganism and spore production. The strain of *Bacillus cereus* (CNRZ 110) was obtained from the Institut National de Recherche Agronomique (France). Spores were kept in distilled water at 4°C. Cells were precultivated at 37°C during 24 hrs in Brain Heart Infusion (Difco). The preculture was used to inoculate nutritive agar plates (Biokar Diagnostics BK021) added with $MnSO_4$ 40mg l^{-1} and $CaCl_2$ 100 mgl^{-1} on the surface area. Plates were incubated at 37°C for 5 days. Spores were then collected by scraping the surface of the agar and suspended in sterile distilled water and washed three times by centrifugation (10000xg for 15 min) (Bioblock Scientific, model Sigma 3K30). The pellet was then resuspended in 5 ml distilled water and 5 ml ethanol. The obtained suspension was then kept at 4°C during 12 hours in order to eliminate vegetative non sporulated bacteria, and washed again three times by centrifugation.

Lastly the final suspension (about 10^{10}spores ml^{-1}) was at last distributed in sterile Eppendorfs microtubes and kept at 4°C.

Thermal treatment of spore suspension. D values in citrate-phosphate buffers adjusted were determined at 95°C with one replicate at each a_w value ranging from 1 to 0.89.

Three solutes (glycerol, glucose, sucrose) were used to adjust the water activity value. The previous molarities of the different solutes were determined using curves from model UNIFAC-LARSEN (2). The heating medium was sterilized by filtration and the a_w values were controlled with an a_w-meter (FA-st1 GBX France Scientific Instrument).

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

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

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

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

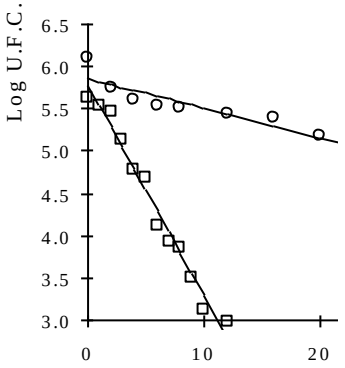
The parameters of the models were estimated by simple linear regression carried out with MINITAB software. The goodness of fit of the model was evaluated by using the per cent variance R^2.

RESULTS

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

0.9, observed $D_{95°C}$ became closed to 11.2 min, 10.5min and 27 min for glycerol, glucose and sucrose respectively.

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



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

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

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

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

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

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

DISCUSSION

It is confirmed that an increase of the water activity produces opposite effects on the apparent heat resistance of spores according whether it concerns the heat treatment either the recovery medium. Inside the investigated water activity range (0.9-1) which corresponds to that of most typical foods, a clear protective effect of an increasing water activity of the heating medium can be observed. For a fixed water activity, the degree of protection depends on the type of used depressor: according to our investigations, sucrose showed a more effective protective effect than glycerol and glucose, which is in agreement with observations by other authors (5, 19, 26, 37). Like in the case of NaCl, the protective influence of an increase of the water activity could partly be balanced by a specific antagonistic and toxic effect of glycerol and glucose . Moreover, the plasmolyse which is partly responsible for the heat protection of spores is limited by the penetration of glycerol and glucose inside cells. This limitation does not exist when the depressor is not uptaken inside cells, which is the case of sucrose. Another explanation for the protective effect of a depressor in the heating medium could be an inhibition of spore germination. Anagnastopoulos and Sidhu (4) observed for *Bacillus stearothermophilus* that the percentage of germination decreases when the water activity decreased and that, at the same water activity, the percentage of germination was lower in a nutrient broth supplemented with sucrose than in a broth supplemented with glycerol. Hydratation of spore protoplast is an important condition of spore activation and initiation of germination. Germination is inhibited in the absence of moisture or in concentrated solution of non penetrating solute. The spores which do not germinate are protected during heat treatment and can germinate and growth during recovery. This explanation is in agreement

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

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

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

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

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

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

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

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 $D_{95°C}$ value vs aw of the heated medium adjusted with glycerol : ○ experimental data, — calculated values

Figure 2b
Bacillus cereus $D_{95°C}$ value vs aw of the heated medium adjusted with glucose : □ experimental data, — calculated values

Figure 2c
Bacillus cereus $D_{95°C}$ 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 $D_{95°C}$ value vs aw of the recovery medium adjusted with glycerol : ● experimental data, — calculated values

Figure 4b
Bacillus cereus $D_{95°C}$ value vs aw of the recovery medium adjusted with glucose : ■ experimental data, — calculated values

Figure 4c
Bacillus cereus $D_{95°C}$ value vs aw of the recovery medium adjusted with sucrose : ♦ experimental data, — calculated values

Figure 5a
Bacillus cereus $D_{95°C}$ value vs aw of both heated and recovery medium adjusted with glycerol : ● experimental data, — calculated values

Figure 5b
Bacillus cereus $D_{95°C}$ value vs aw of both heated and recovered medium adjusted with glucose : ■ experimental data, — calculated values

Figure 5c
Bacillus cereus $D_{95°C}$ value vs aw of both heated and recovery medium adjusted with sucrose : ♦ experimental data, — calculated values

Figure 1

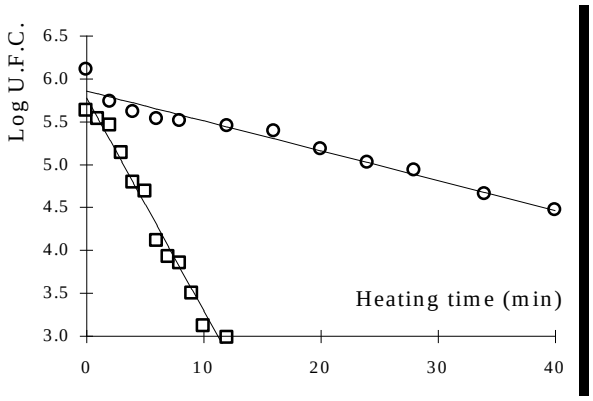

Figure 2a

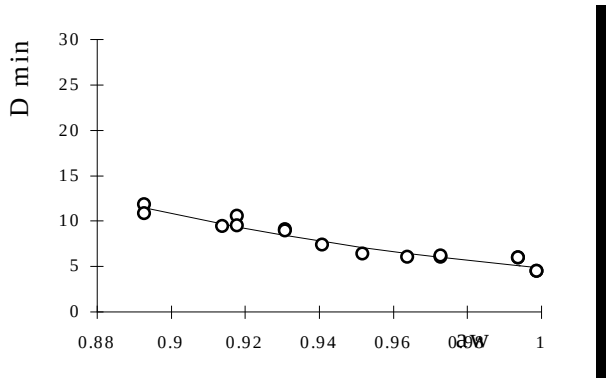

Figure 2b

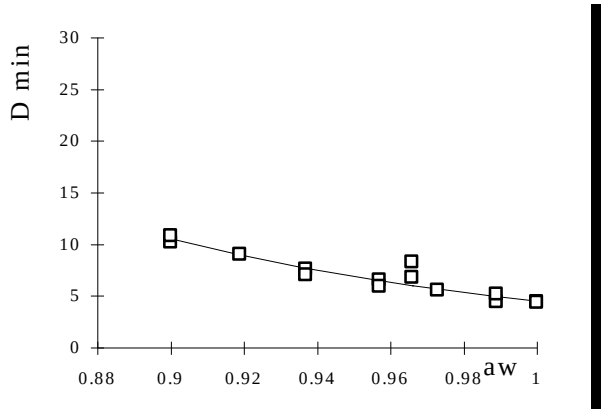

Figure 2 c

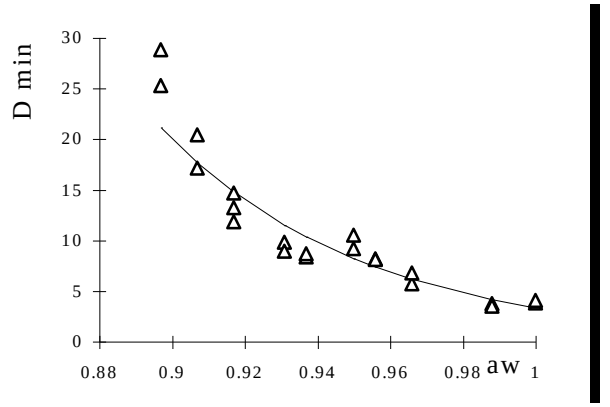

Figure 3

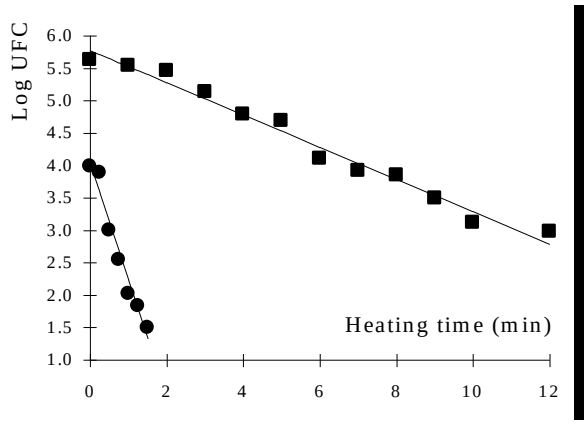
Log UFC
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0
2
4
6
8
10
12
Heating time (min)

Figure 4a

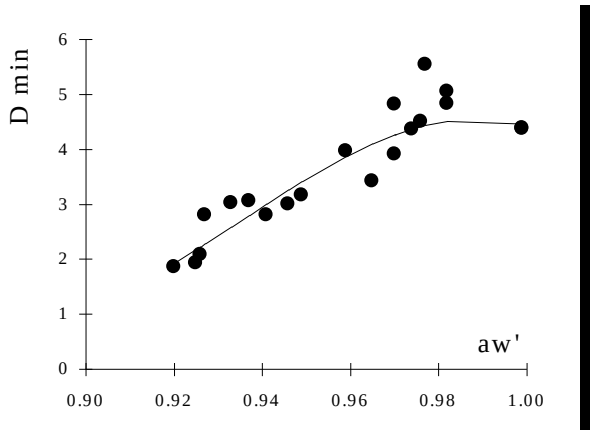

Figure 4b

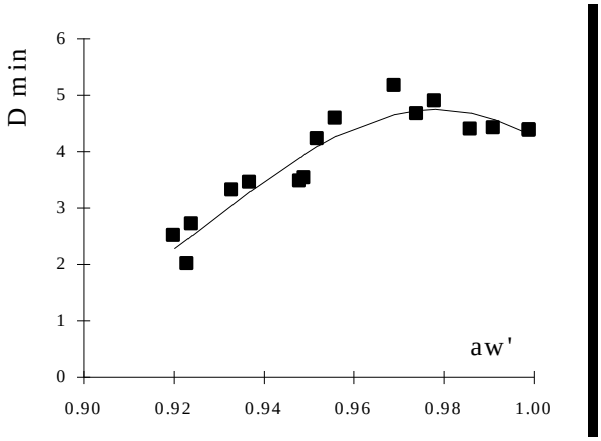

Figure 4c

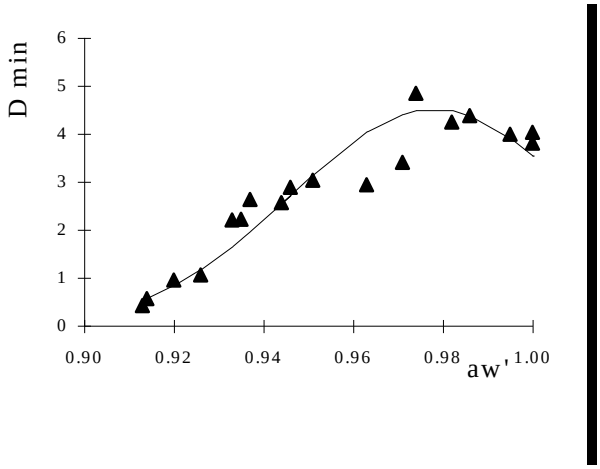

Figure 5a

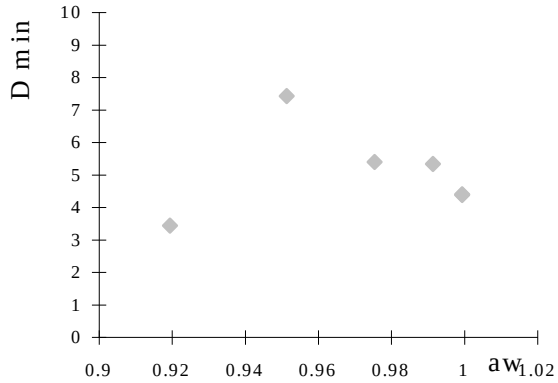

Figure 5b

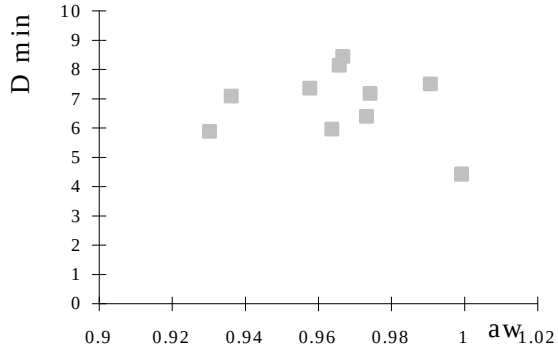

Figure 5c