

The fragile character and structure-breaker role of α , α -trehalose: viscosity and Raman scattering findings

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Abstract. Results of viscosity and Raman scattering measurements on trehalose aqueous solutions are presented. They provide evidence that the trehalose–water system shows a very fragile character, i.e. a great structural sensitivity to temperature and concentration changes. In addition, to get some insight into the effects of trehalose on the hydrogen-bond network of water, we investigate the intramolecular O–H stretching mode, which is particularly sensitive to environmental modifications. For the O–H stretching region analysis, a decomposition of the isotropic spectra into an ‘open’ and a ‘closed’ contribution has been employed. The procedure suggests that trehalose promotes a destructuring effect on the tetrahedral H-bond network of pure water, imposing on adjacent water molecules positions and orientations incompatible with the crystallization process.

1. Introduction

The role of water and the features of aqueous systems are of crucial importance for the maintenance and propagation of life. All life processes depend on the physical and chemical properties of liquid water, such as the hydrogen-bond energy, the spatial disposition of water molecules, its acid–base behaviour and the nature of its microscopic diffusional motions. Moreover, water is essential in maintaining biologically active conformations of nucleotides, proteins and carbohydrates. Simple isotopic substitution with heavy water, for example, is sufficient to alter the balanced coupling between several enzyme-controlled reactions and is extremely toxic. The phase transition from liquid water to ice, being connected with damage of the cellular micro-architecture, is also equated with death. In fact, freezing starts in the extracellular fluids, causing concentration changes and, as a consequence, the alteration of the osmotic equilibrium between the cell and its environment. So, water flows out of the cells with the possibility of mechanical stresses, of physiological and metabolic changes and, finally, of cell shrinkage. Several intercellular solutes, in fact, are released, including nucleotides, amino acids, ions and low-molecular-weight proteins; it is generally believed that the loss of these solutes is responsible for cell death and that the primary cause is an increase in the permeability of the plasma membrane. Many studies have been carried out in order to understand how the delicate equilibrium between cells and tissues and their environment can be preserved. In particular, because the temperature range on Earth lies within the range -40°C to $+40^{\circ}\text{C}$, many organisms are at certain periods subjected to temperatures at which water would normally be frozen. One of the most important parameters as regards surviving in these conditions is the fraction of water remaining in the cells as the temperature is lowered. This is

the unfreezable water which rests within the hydration shell and cannot crystallize; the avoiding of the crystallization process ensures the survival of various organisms such as eggs, cysts, spores and seeds. But organisms have found different mechanisms to protect themselves from freezing based on principles such as undercooling, supersaturation and even vitrification [1]. Freezing avoidance, for example, consists in extending the undercooled region and lowering the crystallization temperature, by means of an increase in solute concentration. Aqueous solutions, in fact, can cool without freezing to temperatures far below the equilibrium freezing point. This metastable state can be stabilized by several strategies which allow the avoidance of contact with or the elimination of the heterogeneous nucleator. For other organisms, survival is connected with the role of certain substances that are able to protect the cells to a substantial degree against osmotic injury. Such a protective effect was first established for glycerol and later for dimethyl sulphoxide; then several other low-molecular-weight species found application as cryoprotectants, for example methanol, mannitol, sucrose and water-soluble polymers. These techniques are widely employed in cryobiology especially for preserving cells, tissues for laboratory use and organs for transplantations, but there exist a large number of animals surviving extreme conditions by taking advantage of this kind of adaptation. In fact, unlike plants, which can exhibit considerable freezing tolerance, animals have developed mechanisms that rely instead on the synthesis of some kind of sugar. It seems that its function is to provide an ordered structure which enables the –OH group on the sugar residues to take up spatial and orientation positions that are not compatible with those of ice. Also in this case, the antifreeze effect is thus related to the inhibition of crystallization. This kind of adaptation is also employed in preserving living cells from the damage caused by extensive dehydration. It is widely shown [2–5] that many lower organisms are able to survive drought conditions and the stress that the loss of water imposes, by synthesizing a certain amount of trehalose (a disaccharide of glucose) and in this way passing into a vitreous state of suspended animation. The vitrification process, incorporating dissolved solutes, avoids osmotic and volume stresses and maintains for decades the structural and functional integrity of organisms. Although these organisms have less complex structures and metabolisms than those of animals and plants, the role of interactions between trehalose and biological membranes at the microscopic level has not been really clarified. Even at low concentrations the disaccharide is capable of affecting the diffusivity parameters of the large water volumes within the hydration shell.

Previous work [6, 7] aimed at elucidating the essential physical mechanisms underlying the effectiveness of trehalose revealed that the hydration number of trehalose molecules does not vary appreciably over the temperature range between 20 and 85 °C. This evidence suggests that the water molecules in the neighbourhood of trehalose are strongly bound to it. Moreover, over the same temperature range, trehalose exhibits a great temperature sensitivity of its conformation.

Quasi-elastic neutron scattering (QENS) measurements [8] showed that the random-jump diffusion of non-hydration water is characterized by a lower self-diffusion coefficient and by a higher residence time in comparison with the values found for pure water at the same temperature. Therefore, trehalose can promote an extensive layer of structured water in its neighbourhood.

The present work shows the results of an experimental study carried out by means of viscosity measurements and Raman scattering on trehalose aqueous solutions at different concentrations and temperatures.

The analysis of the temperature behaviour of the viscosity data indicates a noticeable fragility of the trehalose–water system. This result is also confirmed by the low-frequency Raman spectra which reveal, above and near the glass transition temperature, the absence of a marked boson peak contribution. Moreover, the analysis of the intramolecular O–H stretching

contribution unambiguously shows that trehalose induces extensive destructuring effects on the adjacent water molecules, playing the role of a *structure breaker* of the water H-bond network. These results make plausible the hypothesis that the crystallization process, in the presence of trehalose, is obstructed by the reduction of the amount of freezable water, and could explain the effectiveness of trehalose in maintaining cell life.

2. Experimental set-up

Aqueous solutions of α , α -trehalose were investigated at different temperatures and at concentration c , c being the weight fraction, $c = n_t M_t / (n_t M_t + n_w M_w)$, where n_t , M_t and n_w , M_w are the mole numbers and the molecular weights of trehalose and water, respectively. Ultrapure trehalose was purchased from Aldrich; the solutions were prepared using double-distilled deionized water. Before the measurements, the samples were stored in the dark and left for a long time for equilibration; they were filtered with an Amicon filter of 0.45 μm diameter pores in order to obtain dust-free solutions.

Viscosity measurements were performed by means of standard Ubbelohde viscometers, mounted in a suitable thermostatic bath. The temperature range investigated was $20\text{ }^\circ\text{C} \leq T \leq 85\text{ }^\circ\text{C}$, stabilized within $\pm 0.05\text{ }^\circ\text{C}$. The viscometers were chosen with long flows in order to minimize the kinetic energy correction, and the experimental data were reproducible with an indeterminacy lower than one part per thousand. Auxiliary density measurements, necessary to evaluate the shear viscosity from the kinematic one, were performed using the picnometer technique.

The samples for the Raman light scattering measurements were sealed in an optical quartz cell of inner diameter 5 mm and then mounted in an optical thermostat especially built to avoid any unwanted stray-light contributions. The measurements were performed with a temperature stability better than $0.05\text{ }^\circ\text{C}$. The samples being of high purity, as well as the optical purity of the sample holder, ensured that we collected data with a good signal-to-noise ratio and with high reproducibility. We used, in a 90° scattering geometry, a high-resolution fully computerized Spex-Ramalog triple monochromator. Vertically polarized radiation from an INNOVA 70 Series Ar–Kr-gas mixed laser operating in the 4579–6764 \AA range was used as an excitation source. To reduce the fluorescence we chose the 6471 \AA laser line with a 1 W output. The scattered photons were automatically normalized for the incoming beam intensity in order to ensure good data reproducibility.

To separate the scattering due to the solution from the background, which was almost entirely due to either the Raman scattering or fluorescence of the quartz cell, it was assumed in the removal procedure that the intensity in various spectral regions was, at all temperatures, due entirely to quartz and other types of noise. This is only approximately correct, since there is a detectable sample intensity at this frequency value. However, in comparison to the OH stretching features, the intensity of these spurious contributions is negligible and the assumption is useful. The uncertainty in this correction forms the principal uncertainty in the spectra and was estimated to be less than $\pm 2\%$ of the peak intensity for an I_{VH} -spectrum and less than $\pm 0.5\%$ of the peak intensity for an I_{VV} -spectrum.

Each reported spectrum is the average of different scans. For each isotherm, the individual I_{VV} - and I_{VH} -scans were taken in an alternating sequence to ensure that a definite intensity relationship existed between the final I_{VV} - and I_{VH} -spectra for the same isotherm. Isotropic scattering intensities were calculated from the parallel and perpendicular components of the scattered light using $I_{iso} = I_{VV} - (4/3)I_{VH}$. All of the spectral contributions were normalized to the C–H stretching band; this latter was finally removed from the OH stretching contour.

3. Results and discussion

3.1. The fragile character

3.1.1. Viscosity data. Following the Einstein model in the very dilute limit, the viscosity value for a solution suitable for being modelled as a certain number of hard spheres suspended in a hydrodynamic continuum with viscosity η_0 is $\eta^* = \eta_0(1 + 2.5\phi)$, ϕ being the volume fraction. Because, at low concentration, intermolecular interactions can be neglected, changes in the trehalose molecule shape or strong interactions with the solvent can be hypothesized. It seems that, as shown in figure 1 in which R is reported as a function of T , on increasing the temperature, $R = \eta/\eta^*$ increases from unity more and more. Considering that the two hexapyranose rings constituting the molecule can rotate around the glycosidic linkage, this evolution can be interpreted on the basis of a less and less spherical shape of the molecule. This result is entirely consistent with the finding, evident from previous ultrasonic and photon correlation spectroscopy (PCS) measurements [6, 7], that, on increase of the temperature, trehalose tends to assume an 'open' conformation rather than a folded one.

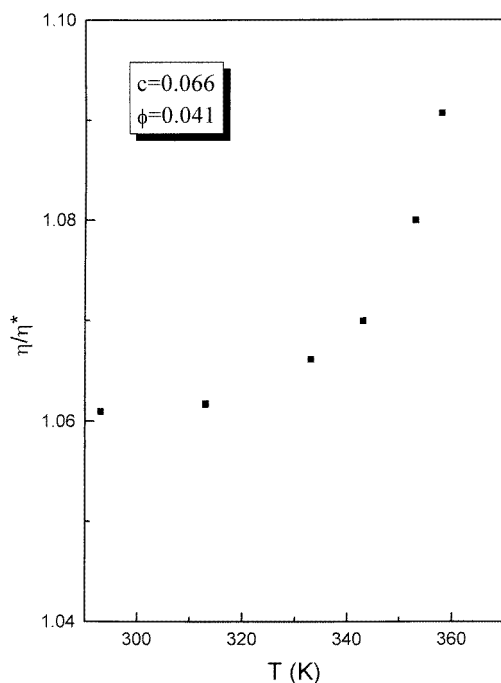


Figure 1. The plot shows the departure from the Einstein model of hard spheres (see the text) for the trehalose–water system.

It is well known that the viscosity behaviour of a liquid in the supercooled region is characterized by an increase of as much as 15 orders of magnitude, up to the glass transition temperature T_g , below which the local equilibration time becomes longer than the experimentally observable time. The range of temperatures in which locally supercooled liquids can be studied is bounded by the T_g -value. For this reason, to compare the viscosity behaviour of different systems, it is necessary to scale them properly with respect to the T_g -value. Following this procedure, Angell was able to classify glass-forming liquids on the basis of their 'strong' or 'fragile' character [9–11]. This classification is based on the choice

of an invariant viscosity at the scaling temperature T_g ($\eta(T_g) = 10^{13}$ P). It can be related to the resistance of the configurational structure near and above the glass transition (with structure indicating short- and medium-range order) to thermal degradation and, hence, related to the potential energy hypersurfaces in configurational space. Fragile liquids (usually ionic liquids without any directional bonds) are characterized by a high density of configurational states and hence by a large degeneracy, which leads to rapid thermal smearing out of the structure with increasing temperature. By contrast, strong liquids can be represented by well-separated minima (a low density of configurational states) and resistance to thermally induced structural changes. In other words, throughout the temperature range, until the glass transition is reached, fragile systems are characterized by an activation energy value which increases as the temperature is lowered towards T_g . In the supercooled region, the more rapid the temperature dependence of the shear viscosity, the more fragile the system.

From a general point of view, the temperature sensitivity to the structural properties can be investigated using the well-known Vogel–Tammann–Fulcher (VTF) [12] function:

$$\eta = \eta_0 \exp \left[\frac{DT_0}{T - T_0} \right] \quad (1)$$

where D , named the *strength parameter*, gives a measure of the degree of ‘strong’ character of the systems. As noted by Johari [13], because of the divergency of D as $T_0 \rightarrow 0$, it is useful to introduce a *fragility parameter*, m , defined in terms of the effective activation enthalpy

$$\frac{\Delta h_{eff}}{R} \equiv \frac{d \ln \eta}{d(1/T)}$$

at T_g [14, 15]:

$$m \equiv \left. \frac{d \log \eta}{d(T_g/T)} \right|_{T=T_g} = m_{min} + \frac{m_{min}^2 (\ln 10)}{D} \quad (2)$$

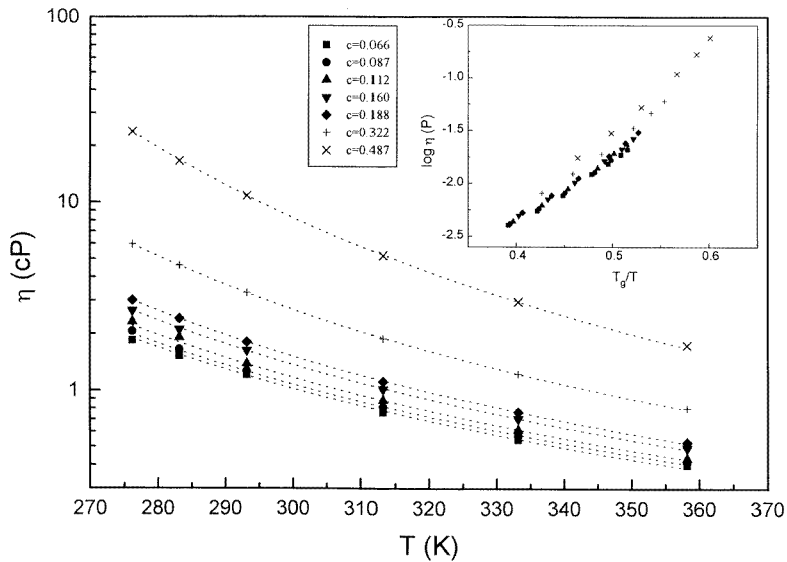


Figure 2. The temperature behaviour of the viscosity data for different weight fraction values. The dashed lines are the fit results for a VTF law.

where $m_{min} = 16$ indicates the value for the strongest systems.

Therefore, viscosity measurements provide a simple criterion capable of providing information on the degree of fragility of a system. To characterize the system as ‘strong’ or ‘fragile’ it is not necessary to perform measurements down to T_g . Knowing the values of T_g of the system and its viscosity at a given temperature, and also far from T_g , it is possible to reconstruct the viscosity profile in the Angell plot and to obtain information on the thermal sensitivity of the system near T_g . This procedure has been applied to many hydrogen-bonded systems such as glycerol, another effective cryoprotectant [9]. Figure 2 shows the temperature behaviour of the viscosity of the trehalose–water system at different concentrations. One can see that the temperature dependence of these data does not obey the Arrhenius law, especially for the most concentrated solution. As a consequence, we adopted the more suitable VTF law, fixing the glass transition temperatures at the values obtained in reference [16]. In the inset of figure 2, viscosity data are reported in an Angell-type plot. Figure 3 reports the values extracted for the fragility parameter as a function of the amount of trehalose. The behaviour exhibited clearly indicates that the degree of fragility decreases as the concentration is raised.

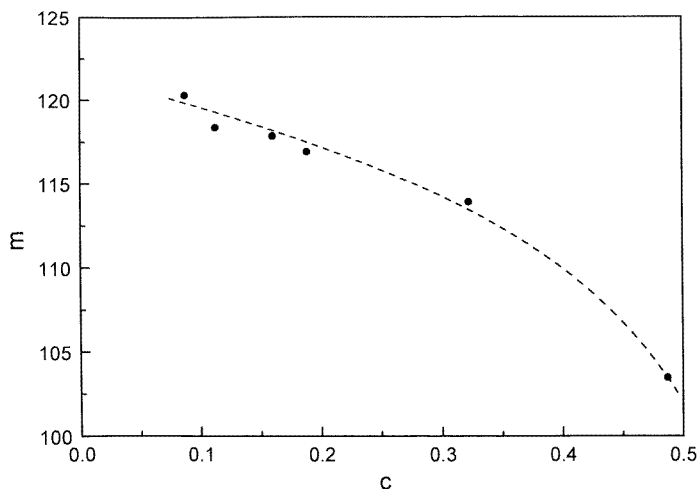


Figure 3. The fragility parameter as a function of the weight fraction.

3.1.2. Low-frequency Raman data. Now we turn to the analysis of the Raman spectra of the trehalose solutions. It is well known that low-frequency Raman spectra ($<200 \text{ cm}^{-1}$) of disordered systems and supercooled liquids usually show two contributions. The first, relaxational in character (i.e. quasielastic scattering), appears at lower, $<15 \text{ cm}^{-1}$, frequencies and reflects relaxations on the picosecond timescale. This contribution increases in intensity at a higher rate than the Bose population factor. The second dominates at frequencies higher than roughly 15 cm^{-1} . It has a peak structure and a spectral density proportional to the Bose factor $(n(\omega) + 1)$, with $n(\omega) = [\exp(h\omega/k_B T) + 1]^{-1}$ for temperatures T below the glass transition temperature. For this reason this is referred to as the boson peak (BP). Both contributions are absent for ordered crystals and their microscopic nature is at present a subject of intensive discussion [17–23].

At intermediate temperatures, the relaxational and the vibrational contributions are comparable in strength and a minimum appears in the spectrum at a frequency lower than that of the boson peak. So, the ratio of the normalized intensity $I_n(\omega) = I/[\omega(n(\omega) + 1)]$

at the minimum to the intensity at the boson peak maximum $R_1 = (I_n)_{min}/(I_n)_{max}$ can be taken as a measure of the relative contribution of the relaxational and vibrational parts of the spectrum.

Recently it was shown [22–27] that R_1 correlates strongly with the fragility of the liquids: in fragile systems the broad quasielastic contribution already dominates the spectra at temperatures around T_g , whereas in strong systems the boson peak dominates even at temperatures far above T_g .

Figure 4 displays the low-frequency Raman spectra of two different trehalose–water solutions, showing a strong quasielastic contribution which dominates even at the lowest temperatures, which were chosen to be one degree above T_g . The absence of the boson peak in the spectra of the trehalose + 20H₂O solution and the more diluted ones leads further to the suggestion of a noticeably ‘fragile’ character of the water–trehalose solutions. A trace of the boson peak can be seen only as a slight shoulder in the spectrum of the trehalose + 2H₂O solution at $T = 3^\circ\text{C}$. This result supports the conclusion based on the viscosity analysis, namely that the decrease of the water content makes the system stronger.

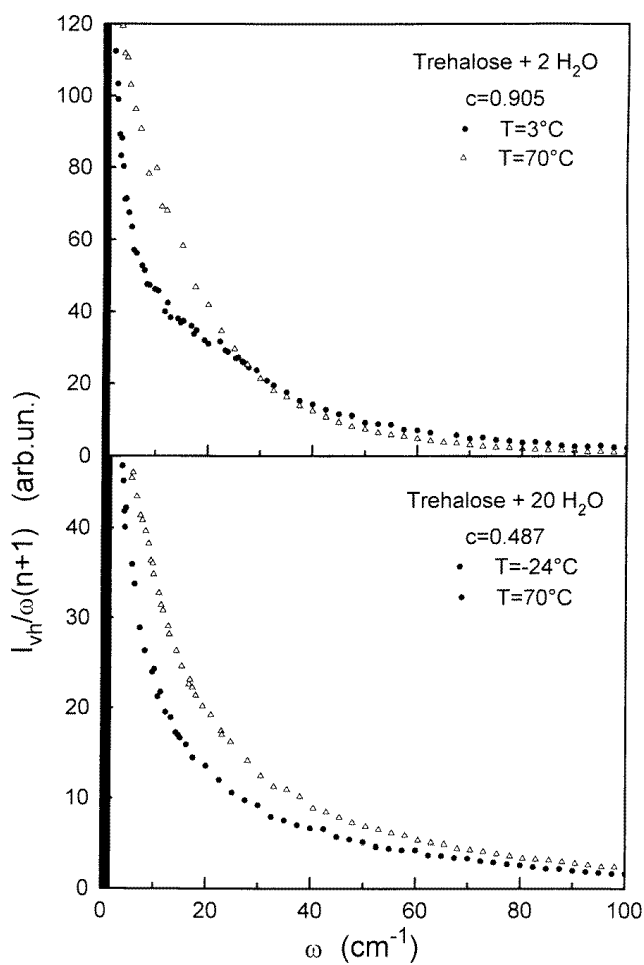


Figure 4. Normalized depolarized Raman spectra for two different concentration values.

3.2. O–H stretching analysis

It is well known that the intramolecular O–H stretching spectral region in aqueous solutions is very sensitive to the local structural environments. As far as pure water is concerned, the existence of an isosbestic point in the isotropic spectrum has suggested to some authors [28–30] the decomposition of each spectrum into an ‘open’ and a ‘closed’ contribution. The ‘open’ contribution, centred at $\approx 3210\text{ cm}^{-1}$, was attributed to the O–H vibration in tetrabonded H_2O molecules, namely to low-density structural arrangements which characterize amorphous ice. The ‘closed’ contribution, centred at $\approx 3420\text{ cm}^{-1}$, corresponds to the distorted O–H vibration of H_2O molecules that do not have a fully developed hydrogen bond. Furthermore, when the temperature decreases, the ‘open’ contribution rises, indicating an enhanced hydrogen bonding [31]. Such an approach has also been applied to sucrose aqueous solutions [32]. The decomposition of each isotropic spectrum into an ‘open’ and a ‘closed’ contribution is reported in figure 5, at three concentration values. For the most diluted sample a third contribution is present, centred at $\approx 3625\text{ cm}^{-1}$; this shoulder is thought to arise from a non-hydrogen-bonded OH stretching vibration and tends to disappear with increasing concentration. Figure 6 shows the ‘open’ fractional integrated intensity, $F(\rho)$, namely the ratio of the integrated intensity of the ‘open’ contribution and the integrated intensity of the total OH stretching contribution, as a function of the molar ratio $\rho = n_T/n_w$, n_T and n_w being the mole numbers of trehalose and water respectively. As can be seen, on decreasing the water content, the ‘open’ fractional contribution markedly decreases. This result suggests that trehalose exerts a destructuring effect on the H_2O tetrabonded network, characterized by spatial and orientation positions that are not compatible with those of ice.

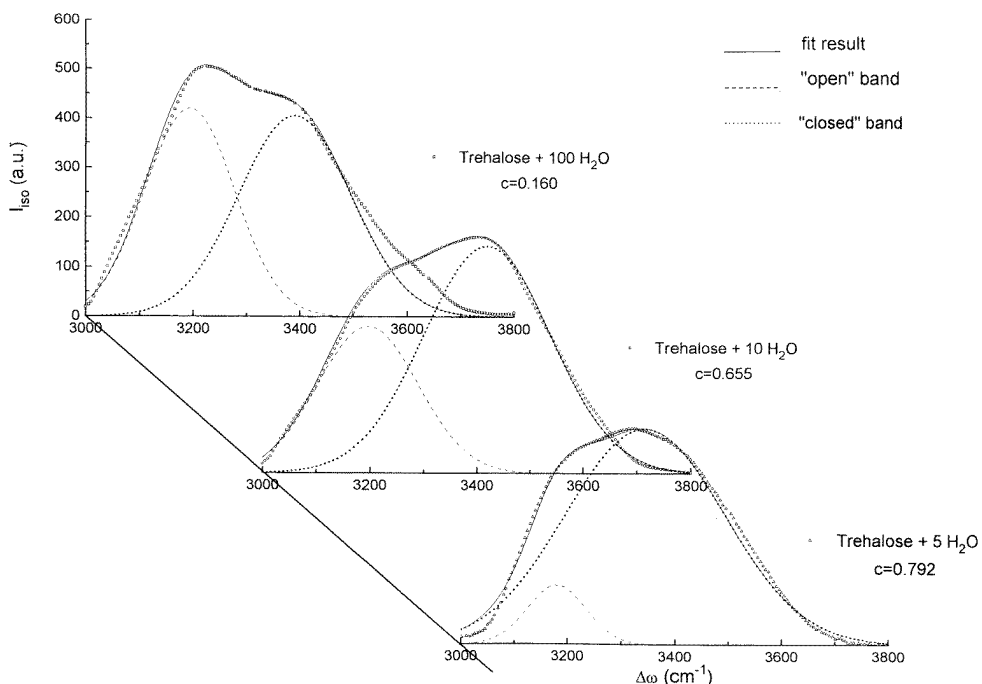


Figure 5. Isotropic spectra, $I_{iso}(\omega)$, of the OH stretching bond of trehalose aqueous solutions for three concentration values at $T = 293\text{ K}$. The decomposition into open and closed contributions is also shown.

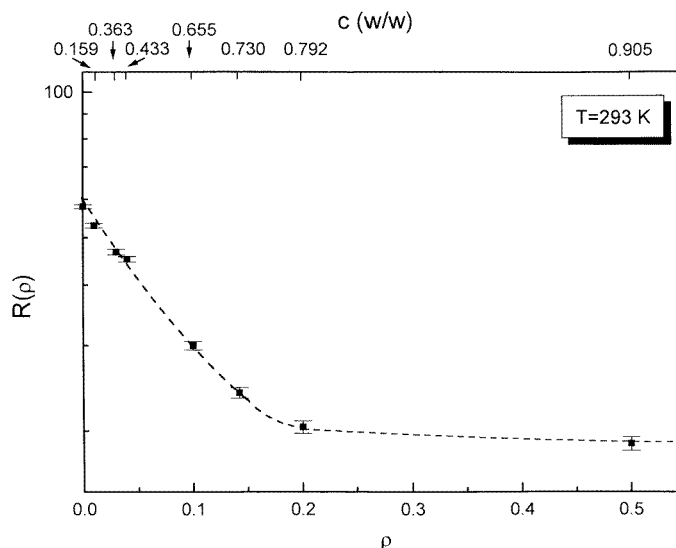


Figure 6. The fractional open contribution, $R(\rho)$, as a function of ρ .

Such a result can be accounted for by considering that the hydrophilic surface of trehalose gives rise to a strongly bound and oriented first layer of water molecules; this latter could give rise to the orientation of the second layer which likewise could influence the third, and so on, inducing the above-discussed destructuring phenomenon.

4. Concluding remarks

The present work shows the large degree of fragility of the trehalose–water system. In the low-frequency Raman spectra, in fact, there is a strong quasielastic contribution and no evidence of a marked boson peak, even at the lowest temperatures near T_g . Viscosity measurements confirm the highly fragile character of these glass-forming systems, showing that the degree of fragility decreases with decrease of the water content. In addition, we have analysed the intramolecular OH stretching contribution, by hypothesizing the existence of ‘open’ and ‘closed’ configurational arrangements contributing to the isotropic spectra. Such an analysis indicates that the presence of trehalose destroys the configurational arrangement of water molecules compatible with the formation of ice.

Considered together, these results support the hypothesis of a great structural sensitivity of trehalose to temperature changes. In particular, trehalose, playing the role of a *structure breaker* of the tetrahedral network of the water molecules, reduces the amount of freezable water and obstructs the crystallization process.

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