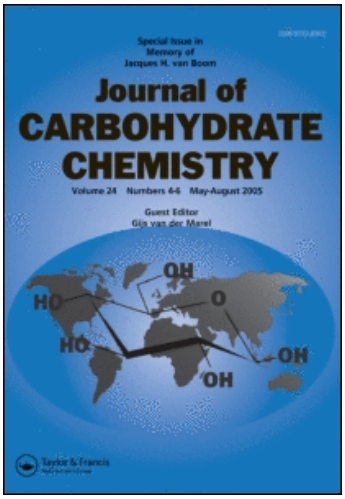

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V. A. Yaylayan[a]; A. A. Ismail[b]
[a] Department of Food Science and Agricultural Chemistry, McGill University - Macdonald Campus, Bellevue Quebec, Canada [b] Steacie Institute for Molecular Sciences National Research Council of Canada Ottawa, Ontario, Canada

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DETERMINATION OF THE EFFECT OF TEMPERATURE ON THE CONCENTRATION OF *keto* FORM OF D-FRUCTOSE BY FT-IR SPECTROSCOPY

V. A. Yaylayan* and A.A. Ismail#

Department of Food Science and Agricultural Chemistry
McGill University - Macdonald Campus
21,111 Lakeshore Road, Ste. Anne de Bellevue
Quebec, Canada, H9X 1C0

#Steacie Institute for Molecular Sciences
National Research Council of Canada
Ottawa, Ontario, Canada K1A 0R6.

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ABSTRACT

FT-IR spectroscopy has been employed in the detection of the carbonyl absorption band centered at 1728 cm^{-1} and assigned to the open form of D-fructose in deuterium oxide or water. Changes in the intensity of the band at 1728 cm^{-1} allowed the monitoring of the concentration of the open form of the *keto* sugar D-fructose at different temperatures and pHs. The concentration of the open form was observed to increase with increasing temperature and was an order of magnitude higher at 80 ^{o}C compared to 30 ^{o}C. The buildup of the open form was found to be extremely rapid. The new equilibrium can be reversed with decreasing temperature with a slight hysteresis. This work demonstrates the potential of applying FT-IR spectroscopy in studying the effect of environmental factors on the level of the open chain form of sugars.

INTRODUCTION

Knowledge of the composition of reducing sugars in solution can have considerable practical and theoretical importance. The physical and chemical properties of these sugars in solution depend on the relative concentrations of different tautomeric forms. Their biological properties can also have similar dependence.[1] In non-enzymatic glycation of proteins and

amino acids (Maillard reaction), the concentrations of open-chain forms might be a crucial factor in determining the rate of the reaction if the mutarotation rate is slower than the reaction rate. The mutarotation of sugars in aqueous solution involves transfer of a proton from an acid catalyst to the sugar, and the transfer of another proton from the sugar to a base catalyst. The reaction starts with an attack on the cyclic form of the sugar by either an acid or a base catalyst, followed by a slow opening of the ring. The acyclic intermediate thus formed plays a major role in the mutarotation reaction.[2] The low concentrations of this form detected in carbohydrate solutions prompted Isbell and Pigman to conclude that the rate of ring closure is faster than the rate of ring opening.[3]

Although most of the tautomeric forms of the reducing sugars in solution have never been isolated, they can be detected by different techniques, and their concentrations in the equilibrium mixture can be measured. Sugars that mainly show interconversions between pyranose anomers, such as glucose, are said to undergo "simple mutarotation"; on the other hand, sugars that also exhibit interconversions between furanose anomers, such as fructose, are said to undergo "complex mutarotation". In both cases, sugars must undergo a ring opening to the open-chain form (also known as *aldehydo* or *keto* forms) in order to mutarotate. The open chain tautomer is usually present in very low concentrations, thus rendering its detection in the equilibrium mixture very difficult.

Nuclear magnetic resonance spectroscopy (NMR) can be used to detect the acyclic forms if they are present in the equilibrium mixture to the extent of 1% or more; however, usually this is not the case for *aldehydo* sugars. The open-chain forms of ketoses were observed only by ^{13}C NMR spectroscopy for solutions of 1-deoxyhexuloses in water[4] and D-fructose in pyridine.[5] In 1979 using ^{13}C NMR, Funcke *et al.*[6] detected the open-chain form of D-fructose in water, but only at 80 ^{o}C and at concentrations greater than 3.7 M. Goux,[7] however, using ^{13}C enriched D-[2-^{13}C]fructose in water, studied the temperature dependence of D-fructose isomerization reactions by ^{13}C NMR, between 17 ^{o}C and 45 ^{o}C and detected the open-chain form at a concentration of 1.5 M and a pH of 8.5. Using ultrahigh resolution ^{13}C NMR and D-glucose (1.4 M in water) C-13 enriched at the carbonyl carbon, Maple and Allerhand[8] were able to detect the *aldehydo* form at temperatures higher than 37 ^{o}C (pH 6.0). The *keto* form of 2-pentuloses can be detected[9] by ^{13}C NMR at room temperature and at low pH since they have relatively high percentages of open-chain forms. ^{13}C NMR spectroscopy is a useful tool to study open chain forms provided ^{13}C-enriched samples are used and the temperature is raised above 37 ^{o}C to increase the content of acyclic forms.

The first study on the application of infrared spectroscopy for the determination of acyclic forms of the sugars was reported by Tipson and Isbell in 1962;[10] they allowed aqueous solutions of several sugars to attain mutarotational equilibrium and the analysis of the dry lyophilized samples indicated that the equilibrium mixture contained some of the acyclic

carbonyl forms of the sugars. Swenson and Barker[11] determined the proportion of the carbonyl form in glyceraldehyde and in several phosphorylated sugars, again using IR spectroscopy, however, they could not detect the *keto* form of D-fructose in solution, since the limit of detection of conventional double-beam IR dispersive instruments was low. The earliest method to provide reliable information on the concentration of acyclic tautomers was polarography. Los *et al.*[12] determined the concentration of the *aldehydo* form of D-glucose in buffered solution from polarographic data to be 0.0026%. However, measurements[13] in unbuffered solution gave the concentration of the *aldehydo* form of D-glucose as 0.04%. Contrary to *aldehydo* forms, the *keto* form gives a distinct UV signal[14] around 280 nm. Although these signals have been used to determine the *keto* content at equilibrium, the reported values are often too high due to unspecificity of this wavelength. Circular dichroism, on the other hand, does not suffer from the same disadvantage; by using the circular dichroism band at 280 nm, the carbonyl content of numerous sugars has been estimated.[15] However, the determined proportions are only approximate, but they are certainly of the right order of magnitude. Until the advent of FT-IR spectroscopy, this method was the only generally applicable method for estimating the proportion of the carbonyl forms in equilibrium. Recently, reversed-phase HPLC has also been used[16] to separate the acyclic form of 1-[(1'-carboxy-2'-indol-3'-yl-ethyl)amino]-1-deoxy-D-fructose (Amadori rearrangement product of tryptophan with D-glucose) from the pyranose and furanose tautomers.

In 1971 Swenson and Barker,[11] after a comparative study of different techniques used in the determination of acyclic forms of reducing sugars, concluded that IR spectroscopy is the most suitable method for this purpose. However, due to the inherent limitations of traditional double-beam dispersive IR spectrometers, this method did not prove satisfactory. The development of FT-IR spectroscopy with its spectral subtraction capabilities and enhanced signal-to-noise ratio allowed the recording of spectra in aqueous solutions and opened new horizons for the study of carbohydrates. Back *et al.*[17] studied the time-dependent FT-IR spectra of pure anomeric glucose and fructose. The spectra obtained revealed many changes in the content as a function of time; fructose, which is known to undergo complex mutarotation, exhibited two types of spectral changes whereas glucose, which undergoes simple mutarotation, showed one type of spectral change. In addition, FT-IR techniques have also been applied to obtain equilibrium constants for mutarotation.[18]

Although FT-IR spectroscopy has been extensively applied in carbohydrate chemistry, the investigations have been generally limited to the following regions of the spectrum: (a) 3600-2800 cm^{-1}, where the CH and OH stretching vibrations take place; (b) 1500-1200 cm^{-1}, containing mainly bands due to the deformational vibrations of groups such as HCH and CH_2OH; (c) 1200-950 cm^{-1}, the C-O stretching region; (d) 950-700 cm^{-1}, containing bands due to the deformational vibrations of COH, CCH and OCH and also including the important

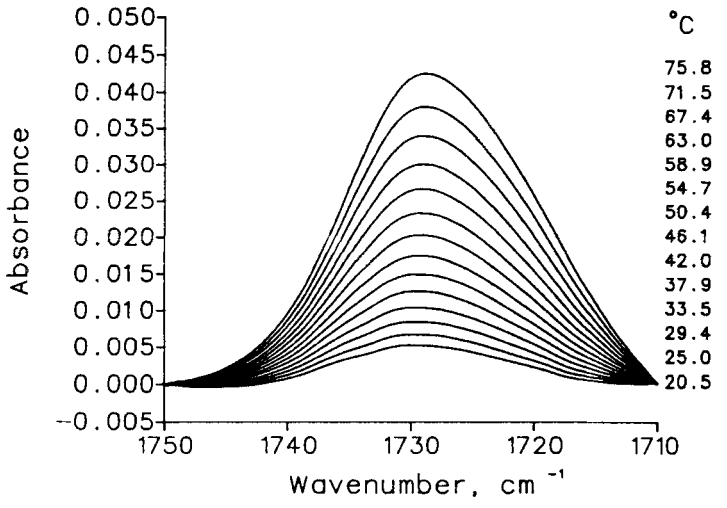

FIG. 1. The increase of the carbonyl band of D-fructose (10^{-3}M) in D_2O with increasing temperature

"fingerprint" or anomeric bands between 930 and 840 cm^{-1} and an appreciable contribution from C-C stretching; and (e) the skeletal region, below 700 cm^{-1}.

It is surprising to find that the carbonyl absorption region between 1800 and 1600 cm^{-1} has not been investigated yet, although Kaanane and Labuza[19] discussed the feasibility of such determinations. One of the reasons for the limited use of the information content from this region is the interference from the strong absorption of water at ~1640 cm^{-1}. The strong intensity of this band requires that very short pathlength (~6 X 10^{-4} cm) IR cells be employed, thus limiting the sensitivity of the measurements. This drawback is particularly serious in view of the fact that the concentrations of the *keto* and *aldehydo* forms of sugars, which have distinct infrared absorption bands in this region, are low at ambient temperatures (~0.001-10%). The extremely high signal-to-noise ratios that can be obtained in FT-IR spectroscopy (approaching 100,000:1) alleviate this problem. In this work, we have utilized FT-IR spectroscopy to detect the *keto* form of fructose and to monitor the effect of temperature on its equilibrium concentration.

RESULTS AND DISCUSSION

Aldehydes and ketones usually exist as tautomeric *keto* and *enol* forms. Aldehydic carbonyl groups absorb at 1740-1720 cm^{-1} and ketone carbonyl groups absorb at 1725-1705 cm^{-1}. The enol groups absorb at 1690-1650 cm^{-1}. The position of the carbonyl stretching band is usually determined by the following factors: (1) the physical state, (2) charge and mass

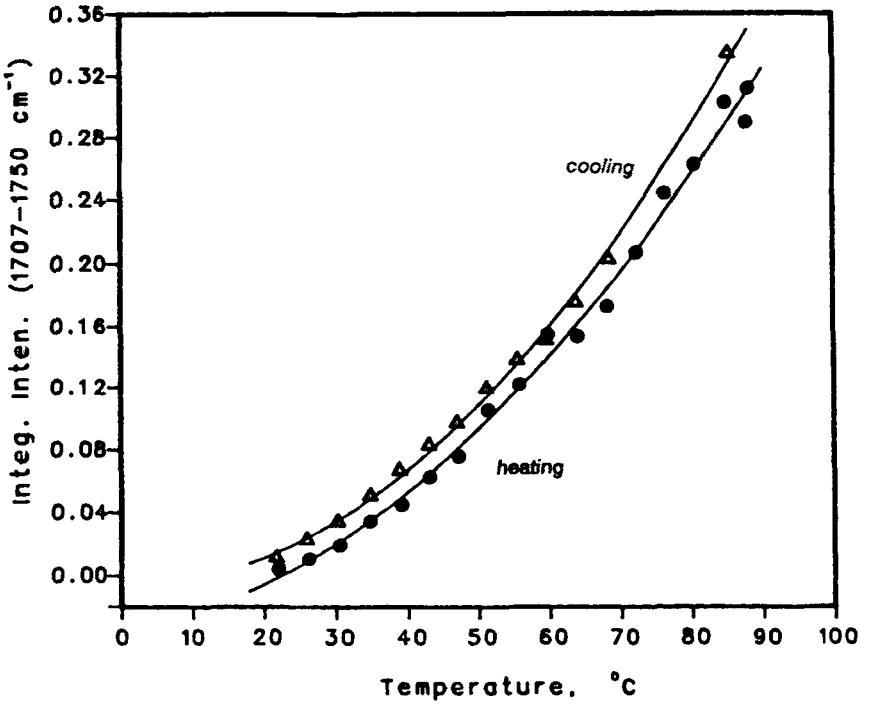

FIG. 2. The plot of integrated intensity of the carbonyl band of D-fructose (in H_2O) vs. temperature

effects, (3) conjugation, (4) intermolecular and intramolecular hydrogen bonding, and (5) ring strain. Hydrogen bonding has special significance for carbohydrates due to the presence of a large number of polar hydroxyl groups and a system of inter- and intra-molecular hydrogen bonds due to them. Hydrogen bonds appreciably influence the physical and chemical properties of carbohydrates. Their role is especially important in the formation of elements of structural orderliness and in separation and stabilization of a given conformational state of the hydroxyl and hydroxymethyl groups.

A saturated solution of D-fructose (after reaching mutarotational equilibrium) in D_2O showed an extremely weak absorption band in the carbonyl region at 1728 cm^{-1}. The intensity of this band was found to be sensitive to temperature. Figure 1 shows the increase in the intensity of the carbonyl band as the temperature was increased gradually from 20.5 ^{o}C to 75.8 ^{o}C. This process was found to be reversible. Figure 2 shows a plot of the integrated intensities of the carbonyl peak as a function of temperature; a slight hysteresis upon cooling is observed. It should be noted that the carbonyl peak reaches its maximum height in less than 10 min,

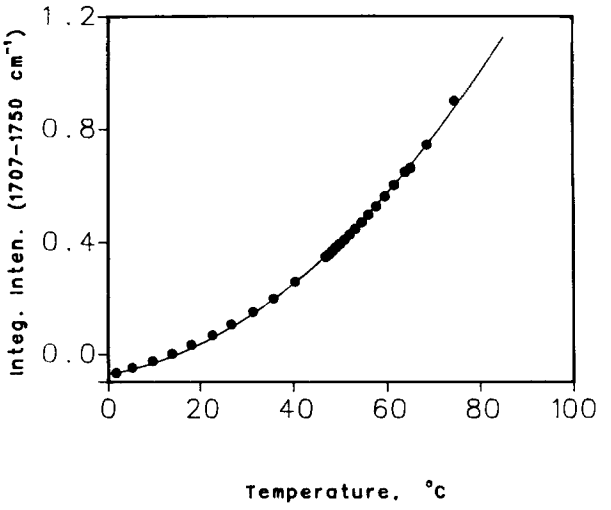

FIG. 3. The plot of integrated intensity of the carbonyl band of D-fructose (1 M) in D_2O vs. temperature (cooling cycle)

indicating that the new equilibrium concentration of the *keto* form at any given temperature is achieved very rapidly.

Plotting the integrated intensities of the carbonyl band (1710 - 1750 cm^{-1}) versus temperature (see Figure 3) roughly defined a parabolic curve which could be fitted into a second degree quadratic equation (see Table I). This equation relates the intensity of the carbonyl band (amounts of open chain form) to the temperature. The first derivative (the change in intensity per unit temperature) of this equation, was defined by a first degree quadratic equation whose slope represents the average change in the intensity of the carbonyl band per degree centigrade (between 30-80 ^{o}C). The values relative to pH 7 (in D_2O) are termed Relative Average Change in Intensity (RACI) per degree and are reported in Table 1.

The result indicates that the increase in the amount of open chain form for every degree rise in temperature was the same as the amount of decrease of the open chain form for every degree fall in temperature. In addition, at basic pH the RACI per degree (both in heating and cooling cycles) was 1.6 times that at neutral pH. Changing the solvent from D_2O to H_2O also increases the intensity of the carbonyl absorption by a factor of 1.2.

The fact that RACI per degree was the same during the heating cycle (ring opening) and the cooling cycle (ring closing), indicates that equilibria among different tautomers are achieved within the time frame of FT-IR. In addition, the integrated intensity of the carbonyl band (in D_2O) increased approximately 8-fold at pH 7 and 9-fold at pH 8 when the temperature

TABLE 1. Relative average changes in intensity (RACI) of the carbonyl band of D-fructose per degree, between 30 and 80 ^{o}C, in D_2O

pH	Curve fitting formulae	S.D.	RACI/degree
7^{h}	$Y = (0.15x10^{-3})X^2 + (0.11x10^{-2})X - 0.038$	0.002	1
7^{h*}	$Y = (0.35x10^{-4})X^2 + (0.82x10^{-3})X - 0.036$	0.0090	1.2
7^{c}	$Y = (0.13x10^{-3})X^2 + (0.28x10^{-2})X - 0.071$	0.0086	1
7^{c*}	$Y = (0.45x10^{-4})X^2 + (0.61x10^{-4})X - 0.008$	0.0047	1.2
8^{h}	$Y = (0.19x10^{-3})X^2 + (0.56x10^{-2})X - 0.17$	0.0021	1.6
8^{c}	$Y = (0.27x10^{-3})X^2 - (0.14x10^{-2})X + 0.83$	0.012	1.6

* *in H_2O*
h heating cycle, c cooling cycle
Y Integrated intensity, X Temperature
S.D. standard deviation

TABLE 2. Changes (fold) in the integrated intensities of the carbonyl absorption band between 30 - 80 ^{o}C

Solvent	pH 7 (h)	pH 7 (c)	pH 8 (h)	pH 8 (c)
D_2O	+ 7.78	- 7.58	+ 8.84	-9.35
H_2O	+12.5	-8.30		

+ increase, - decrease
c cooling, h heating

was raised from 30 to 80 ^{o}C, whereas in H_2O the increase in the intensity was around 12-fold (see Table 2).

The effect of solvent

Reactions subject to general acid-base catalysis such as mutarotations proceed more rapidly in water than in deuterium oxide. This difference arises from a combination of kinetic and solvent isotope-effects. The latter effect can arise when the isotopic compound is used both as a reactant and as a solvent. Change of solvent from H_2O to D_2O causes changes in the degree of ionization and solvation, both of the reactants and of the catalyst. The ion product of deuterium oxide[20] is 8.91×10^{-16}, and that of water[21] is 6.76×10^{-15}. Numerous studies have been conducted on the mutarotation of sugars in deuterium oxide and in water. From such studies, Challis *et al.*[22] concluded that the acid-catalyzed mutarotation involves a rapid, equilibrium exchange between the deuterium or the hydrogen atoms of the solvent and the anomeric hydroxyl group, followed by the breaking of the anomeric O-H bond in water, and of the O-D bond in deuterium oxide, with simultaneous ring opening in both cases, assisted by the conjugate base of the acid catalyst. The result obtained in this study corroborates the above conclusion that ring opening and closing is faster in water than in deuterium oxide.

CONCLUSION

Evidence for the presence of the *keto* form of D-fructose in solution has been provided by methods such as polarography and ^{13}C NMR spectroscopy. The advantage of employing FT-IR spectroscopy in the study of *keto* sugars is twofold. First, the *keto* form can be detected in low concentrations (>0.05%) without the need to utilize ^{13}C-enriched compounds as is the case in NMR studies. Second, the time scale of IR is much faster and thus, in principle, rapidly exchanging conformers may be more easily detected by IR spectroscopy provided their concentrations fall within the sensitivity limits of the IR spectrometer. The current studies have indicated that the steady state concentration of the open form of fructose increases with increasing temperature. This increase in the steady state concentration, which is completely reversible with decreasing temperature, can either be a result of an increase in the rate of formation of the *keto* form or a decrease in the rate of ring closure or a combination of both. We are currently working on the determination of the approximate molar absorptivity of the *keto* form in order to determine its concentration in solution. This data can be very useful for the determination of the relative concentrations of the cyclic and the acyclic forms at a given temperature. In addition, a systematic examination of different *aldehydo* and *keto* sugars in different solvents is also underway. This will add to the scarce data available on the acyclic

forms of sugars, FT-IR spectroscopy being one of the simplest methods for obtaining direct structural information at the molecular level.

EXPERIMENTAL

D-Fructose was obtained from Sigma Chemical Co. Water-d_2 was obtained from MSD Isotopes. D-Fructose solutions were made up in concentrations ranging from 10^{-3} M to saturated in water-d_2 or water. The solutions were left to stand for a minimum of 48 h at room temperature prior to FT-IR measurements. The pH values are corrected pD values (pH = pD + 0.4).

Temperature Studies:

A fructose solution in D_2O was placed in a CaF_2 IR cell with a 50-um Teflon spacer. The temperature of the sample was regulated by placing the IR cell in a temperature-controlled cell holder. Infrared spectra were recorded on a Digilab FTS-60 Fourier-transform spectrometer, purged with dry nitrogen and equipped with a deuterated triglycine sulfate (DTGS) detector. A total of 128 scans at 4-cm^{-1} resolution were coadded. Spectral subtraction of the solvent and data reduction were carried out with programs developed at the National Research Council of Canada.

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