# Comparison between Two Methods To Measure Translational Diffusion of a Small Molecule at Subzero Temperature

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The diffusion of a small molecule is one of the determining factors that control evolution in food products during storage at low water content or in the frozen state. The diffusion of a small molecule, fluorescein, in highly viscous media (concentrated sucrose solutions) has been studied, using two tracer techniques: a concentration profile method and fluorescence recovery after photobleaching (FRAP). In the large temperature range studied, from −10 to 20 °C, the diffusion coefficients of fluorescein, obtained with the two methods, are similar. FRAP can be used only for transparent samples, but it is a rapid method to determine low diffusion coefficients. The concentration profile method can be used for diffusion experiments in nontransparent samples, containing ice for example.

**Keywords:** *Sucrose solutions; FRAP; concentration profile; diffusion*

## INTRODUCTION

As the stability of a food determines its preservation for a long period, it is important to protect commercially available foodstuffs against degradation. The molecular mobility of food components is a determining factor of the kinetics of physical, chemical, or enzymatic reactions that can change food. The determination of the translational diffusion of trace amounts of low molecular weight molecules in food with low water content is thus of practical and theoretical interest to control the stability of food products. The main purpose of this study is to measure the diffusivity of small molecules in viscous media, at temperatures close to the glass transition temperature of the sample. Under these conditions, the diffusion coefficients are low, compared with the diffusion at temperatures much above the glass transition temperature, even if the diffusant is a small molecule such as a monocarbohydrate. To model the translational diffusion processes of a small molecule in low-water food, the diffusion coefficient must be known, but its experimental determination in real food is usually intricate. Where it is possible, model systems allowing transfer of the results to real food are advised for a good understanding of the processes. Solutions of sucrose in water are used as a food model system in our investigations at low temperatures because sucrose is a common component in frozen food products, particularly found in ice cream, for example.

A variety of techniques have been used to measure the diffusion of small molecules in polymer solutions and in food products. Sorption and permeation, for example, were originally used to measure the diffusion in polymer membranes or films and remain popular for studying practical problems. Both techniques are valid for the determination of diffusion coefficient as low as $10^{-16}$ m$^2$ s$^{-1}$, but in practice they are not used for the study of media like viscous carbohydrate solutions.

NMR spectroscopy reveals useful information about molecular structure, chemical reaction rates, and diffusion processes. Recent developments in NMR opened several new areas for food scientists, such as solid-state NMR, NMR imaging, metabolic NMR, and two- and three-dimensional NMR (Hemminga, 1992). Norwood (1993), for example, reported results about $D_2O$ diffusion measured by a pulsed gradient spin echo NMR technique (PGSE NMR) in a piece of celery. The PGSE NMR measures a true self-diffusion coefficient without any concentration gradient or any labeling of the material. This technique is commonly used for the diffusion of water. It allows for the measurement of small coefficients down to $10^{-14}$ m$^2$ s$^{-1}$, but this technique is often used for the moment with efficiency, in the case of media containing water, to water diffusion coefficient evaluation. The proton NMR can be carried out in low water content samples with difficulty, but another NMR technique may be an interesting choice with the development of the sample preparation.

In this study, two well-known tracer techniques that allow the determination of the translational diffusion coefficient have been used and compared in food model systems. Both were used to study the mobility of a small molecule, the "diffusant", in carbohydrate solutions, the "matrix". One of these techniques was fluorescence recovery after photobleaching (FRAP), which monitors the molecular mobility of a fluorescent diffusant. The other was based on the evolution with time of a step concentration gradient of diffusant in the matrix. The shape of the concentration profile varies with time according to Fick's second law and is analyzed with a relation given by Crank (1975). In both techniques, the same diffusant was used.

FRAP is a holographic grating technique similar to forced Rayleigh scattering (FRS). Both techniques measure the tracer diffusion coefficient of labeled diffusants in polymer solutions. FRS can measure diffusion coefficients down to about $10^{-20}$ m$^2$ s$^{-1}$ and was first applied to polymer systems by Hervet et al. (1978) to study the diffusion in solutions of chain polymer labeled with a photochromic dye molecule. The photochromic dye present in the sample undergoes, under laser illumination, either a trans−cis isomerization or a ring opening and closing or a hydrogenation of a carbonyl carbone. The photochromic dyes used with

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FRS are often nonsoluble in water or relatively big molecules. Moreover, in the case of very low diffusion coefficients, the problem with such probes is the lifetime of the excited state of the dye molecule. In FRAP, the labeling is achieved by photobleaching of a fluorescent dye; this photomodification is irreversible, contrary to those used in FRS. Both techniques measure the diffusion coefficient of the dye molecules without a probe gradient. Futhermore, using fluorescent dyes gives a broader choice of probes. FRAP is simple both in concept and in practice. A small region of a sample originally containing uniformly dispersed mobile fluorescent molecules is exposed to a brief intense pulse of light, thereby causing irreversible photochemical bleaching of the fluorophore in that region. The diffusion coefficient is determined by monitoring the recovery of fluorescence in this region as a function of time. The FRAP technique has been extensively used to study the mobility of molecules in various media, such as macromolecules in polymer solutions or melts (Davoust et al., 1982; Bu and Russo, 1994) the dispersion of spheres (Imhof et al., 1994), and proteins or lipids, in a variety of cells and tissues (Yguerabide et al., 1982). The FRAP technique is very suitable to our problem because it allows the determination of diffusion coefficients from $10^{-9}$ to $10^{-17}$ $m^2$ $s^{-1}$ with typical uncertainties of 1−10%.

The FRAP method provides information on the displacement of molecules over distances of the order of some micrometers, allowing the determination of low diffusion coefficients within an hour, for example, if the diffusion coefficient value is around $10^{-15}$ $m^2$ $s^{-1}$. The drawback of the technique is that the sample must be optically transparent to evaluate the fluorescence; for example, the study of samples containing ice is not possible with the FRAP technique. So the concentration profile method complements the experimentation because this technique monitors the probe diffusion on a macroscopic scale and allows the study of the influence of ice on the diffusion.

The concentration profile method has been shown to be very effective in determining diffusion coefficients, for example, the diffusion of palmitic acid in paraffin oil (Naesens et al., 1981) or of volatile components in carbohydrate solutions (Bettenfeld, 1985). When using this techniques, these authors had to cut some gel slides about 2 mm thick and then weigh them before measuring the diffusant concentration. When the translational diffusion coefficient value is low, the concentration profile method requires a time sufficiently long to monitor the diffusant advance on a distance which is measurable by the probe detection system. Because this manipulation was very lengthy in practice, we have chosen to change it slightly.

The two techniques, concentration profile and FRAP, were both tested on the diffusion of the same molecule: fluorescein in the same solutions containing sucrose at high concentration and agar−agar for a large range of temperatures from −10 to 20 °C.

## EXPERIMENTAL PROCEDURES

**Materials.** The matrix of diffusion was made with coarse grained sucrose dissolved at different concentrations from 43.5% to 65.3% in distilled water. To carry out the experiments with greater convenience, the diffusion matrix was mixed with agar−agar in powder at the concentration of 1% to make a gel.

The diffusant molecule, fluorescein (disodium salt, Kuhlmann), was used in solution in distilled water at a concentration of 30 μM for the concentration profile technique and at a concentration of 3 μM for the FRAP technique. The probe solution had a pH of 7.

**Methods.** *Concentration Profile Method.* A sucrose solution was prepared at the required concentration. It was then divided in two, and agar−agar powder was added to the two solutions in the same quantity. One sample was mixed with the fluorescein solution to obtain a concentration of 30 μM; the other one remained without probe. The solutions were heavily agitated for more than 20 min at 80 °C in a double boiler until homogenization. The diffusion experiments were performed in plastic transparent tubes of 4.8 mm i.d. and about 150 mm long. The solution without fluorescein was transferred first, using a syringe, into a tube that was closed at one end. The gel, about 70 mm long in the tube, was kept in an upright position and inserted into a glass tube immersed in a melting ice bath. After cooling, when the gel was formed, the solution containing the fluorescein was poured into the tube in the same way as the solution without tracer. The tube was closed at the other end. At the reference time $t = 0$, all of the tubes were horizontally put in temperature-controlled baths; the fluctuations in temperature in the sample were less than ±0.5 °C around the set-point temperature. For each concentration and temperature, three samples were prepared. The samples remained in the controlled-temperature bath for a few days up to 45 days, for example when the experiment was carried out at −10 °C, because the diffusion coefficient was very low at this temperature. After a time depending on temperature, the diffusion profile was determined with a spectrophotometer to obtain the concentration profile curve. The plastic tube was put in a flying spot scanner (CS 9000, Shimadzu), and the optical density, proportional to the fluorescein concentration, was recorded along the length of the tube at a wavelength of 485 nm. Spectrophotometric measurements were carried out at room temperature. The total duration of the measurement never exceeded 7 min, to minimize the changes in the concentration profile. Thirty data were collected on the concentration profile curve to calculate the diffusion coefficient.

*Fluorescence Recovery after Photobleaching.* A small diffusion coefficient can be measured by creating a periodic pattern with very small repeat distances on the order of micrometers. Periodic fringes were created by crossing two laser beams of equal intensity in the sample, creating an interference pattern. This was achieved by splitting a coherent laser beam into two nearly equal intensity beams, which were recombined at an angle $\theta$ in the sample. In the crossing region, interference effects produce a sinusoidal light intensity pattern with a period $d$.

$$d = \lambda/[2 \sin(\theta/2)] \quad (1)$$

$\lambda$ is the laser wavelength and $\theta$ the angle formed by the two beams focused in the sample. Before the diffusion measurements, the beams were focused through a microscope objective and the period $d$ was determined by projecting the interference fringes on a paper calibrated with a micrometer. During a short time, smaller than 0.2 s, the beams were sent into the sample with an intensity of 120 mW. Some of the fluorophores in the bright areas of the interference pattern were photobleached, creating a nonuniform concentration distribution of the dye. The recovery of fluorescence within the photobleached area was monitored using the same fringe pattern as the photobleaching one with the same laser beams attenuated by a factor $10^3$. The fluorescence light was detected by a photomultiplier (PM) through an optical band-pass filter centered at 520 nm to avoid stray light. The intensity $I(t)$ of the fluorescence light was stored in a computer. The main components of the FRAP technique used in our experiment are shown in Figure 1.

The sample, a sucrose solution with fluorescein (3 μM) and 1% agar−agar was prepared in the same conditions as the tracer solution in the concentration profile method. The probe concentration was in a lower amount because the fluorescence light measurement is more efficient than a colorimetric method. The solution was introduced into a standard spectrophotometric tube. The fluorescence excitation wavelength was 458 nm, and the emission wavelength was 520 nm. The

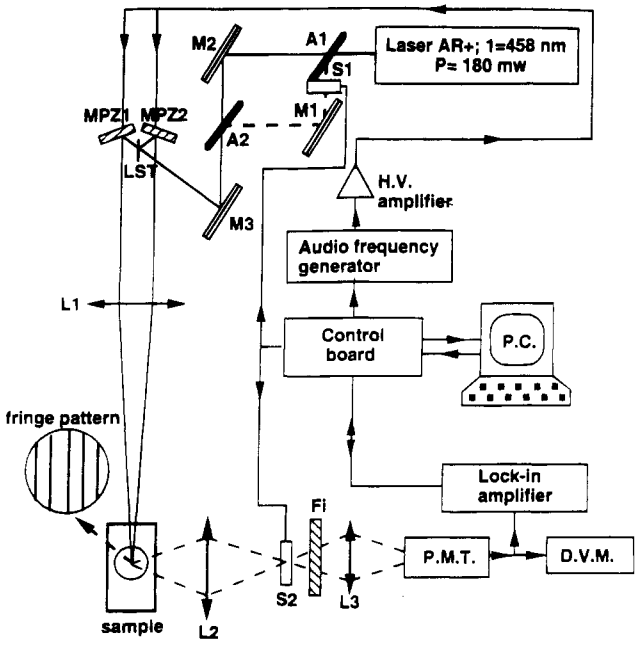



**Figure 1.** Schematic of the experimental setup used in the FRAP technique.

standard spectrophotometric tube containing the sample was closed and a precision thermic resistance to measure the temperature in the sample was introduced through the stopper. The sample was set in a closed box with two transparent windows: one for the laser beams and the other to read the fluorescence light. The side of the tube in contact with a copper sheet was coated with silicon grease to increase the thermal exchange. A Peltier effect system maintained the temperature of the sample as required, and the temperature control was carried out with an accuracy of about ±0.2 °C.

For every FRAP experiment, the measurement was repeated at least five times, moving slightly the sample every time; the data were added to increase the signal over the noise ratio.

## RESULTS AND DISCUSSION

**Diffusion Coefficient Calculation.** Time dependent diffusion in one dimension with a constant diffusion coefficient is described by Fick's second law:

$$\delta C/\delta t = D(\delta^2 C/\delta x^2) \tag{2}$$

$C$ is the fluorescein concentration at distance $x$ (in m) after time $t$ (in s) and $D$ the diffusion coefficient in $m^2$ $s^{-1}$.

For the case of diffusion into a semi-infinite medium with a constant concentration $C_0$ of fluorescein in the part with tracer ($x < 0$), and an initial concentration of zero in the other part ($x > 0$), as realized in the concentration profile method, the solution to Fick's law is given by Crank (1975):

$$\frac{C(x,t)}{C_0} = \frac{1}{2}\,\mathrm{erfc}\left(\frac{x}{2\sqrt{Dt}}\right) \tag{3}$$

$C(x,t)$ is the fluorescein concentration at distance $x$ after time $t$, $D$ the diffusion coefficient, $C_0$ the initial concentration in fluorescein, and erfc the complement of the error function (erfc = 1 − erf).

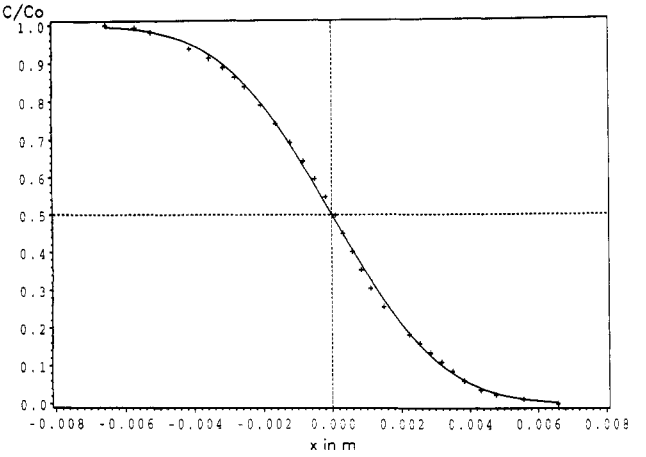



**Figure 2.** Relative concentration of fluorescein ($C/C_0$) versus distance $x$ obtained with the profile technique (57.5% sucrose at −5 °C): (×) experimental values; (—) result of the fit to eq 5.

In a preliminary experiment, we had checked that, within the concentration range of fluorescein we used, the absorbance was proportional to the fluorescein concentration. The 30 data collected on the spectrophotometric curve were translated into a concentration profile. The diffusion coefficient was determined by fitting these data to the theoretical profile given by eq 3. According to eq 3, the origin $x = 0$ was set at the point where the concentration $C_{(0,t)}$ was half of $C_0$. The diffusion coefficient $D$ was determined by using the SAS nonlinear regression procedure (SAS, 1989) for the 30 data. An example of a theoretical profile and experimental data is shown in Figure 2. The diffusion coefficients were obtained with uncertainties in a range of 5−20%.

For FRAP measurements, the diffusion coefficients are determined by using eq 2, with different initial conditions. At $t = 0$, just after the bleaching, the dye concentration distribution is given by

$$C = C_0 \exp[i(2\pi/d)x] \tag{4}$$

with $d$ being the interference fringe period. From eqs 2 and 4, one can write

$$\frac{dC}{dt} + D(4\pi^2/d^2)C = 0 \tag{5}$$

and the solution of eq 5 is

$$C = C_0 \exp[-D(4\pi^2/d^2)t] \tag{6}$$

The intensity $I(t)$ of the fluorescence light, stored in a computer, was analyzed by fitting it to

$$I(t) = A \exp[-(t/\tau)] + B \tag{7}$$

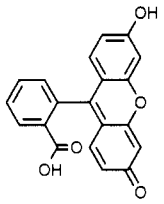
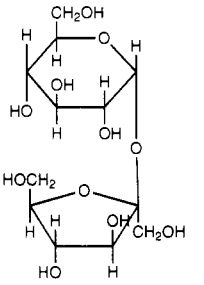
where $t$ is time and $\tau$ a relaxation time of diffusion. $A$ and $B$ are determined from the fluorescence recovery curves, $A$ being the maximum amplitude of the fluorescence intensity just after the photobleaching and $B$ the fluorescence intensity value before the photobleaching.

$\tau$ was evaluated from the recovery graph and its fitting with eq 7, and the diffusion coefficient $D$ was

**Table 1. Values of the Diffusion Coefficient of Fluorescein in Sucrose Solutions at Various Temperatures and Concentrations Obtained with the FRAP and Profile Techniques**

| matrix | FRAP results | | profile results | |
|---|---|---|---|---|
| | $T$, °C | $D \times 10^{-12}$ m$^2$ s$^{-1}$ | $T$, °C | $D \times 10^{-12}$ m$^2$ s$^{-1}$ |
| 43.5% sucrose + 1% agar–agar | 20 | 49.99 ± 7.1 | 20 | 50.21 ± 7.0 |
| 57.5% sucrose + 1% agar–agar | −5.1 | 1.274 ± 0.1512 | −5 | 1.040 ± 0.106 |
| 57.5% sucrose | −5.15 | 1.532 ± 0.148 | no determination without gel | |
| 57.5% sucrose + 1% agar–agar | −10.0 | 0.9139 ± 0.011 | −10 | 0.928 ± 0.102 |
| 57.5% sucrose | −10.1 | 0.9232 ± 0.039 | no determination without gel | |
| 65.3% sucrose + 1% agar–agar | −4.9 | 0.3323 ± 0.0476 | −5 | 0.337 ± 0.029 |
| 65.3% sucrose + 1% agar–agar | −10.1 | 0.14925 ± 0.01883 | −10 | 0.139 ± 0.024 |

**Table 2. Comparison of the Molecular Parameters of Sucrose and Fluorescein: Molar Weight, Molar Volume, Hydrodynamic Radius, and Maximum Length of the Molecule**

| fluorescein | | | sucrose | |
|---|---|---|---|---|
| structure | data | parameter | data | structure |
|  | $C_{20}H_{12}O_5$ | formula | $C_{12}H_{22}O_{11}$ |  |
| | 332.3 g mol$^{-1}$ | molar weight | 342.3 g mol$^{-1}$ | |
| | 315 cm3 g$^{-1}$ mol$^{-1}$ | molar volume[a] | 313.9 cm$^3$ g$^{-1}$ mol$^{-1}$ | |
| | 10.4 Å | maximum distance[b] | 11.0 Å | |
| | 5.02 Å[c] | hydrodynamic radius | 4.9 Å[d] | |
| | 488[f] | diffusion coefficient,[e] $D \times 10^{-12}$ m$^2$ s$^{-1}$ | 520[g] | |
| | 510 ± 20[h] | | 521[i] | |
| | 513 ± 15[j] | | 540[k] | |

[a] Le Bas (1915). [b] Determined with Molecular Advanced Design program (Oxford Molecular). [c] FRAP measurements by Mustafa et al. (1993). [d] Viscosity measurements by Mathlouthi (1980). [e] The fluorescein data were obtained by the FRAP method, and the sucrose data were determined with a Stokes cell. [f] Bu and Russo (1994). [g] Loncin (1976). [h] Mustafa et al. (1993). [i] Weast et al. (1984). [j] De Smedt et al. (1994). [k] Chandrasekaran and King (1972).

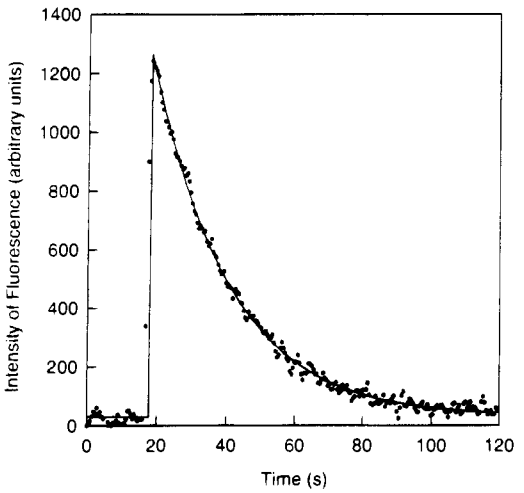



**Figure 3.** Relaxation of the fluorescence intensity versus time obtained in a FRAP experiment (57.5% sucrose at 0.3 °C): (●) experimental values; (—) result of the fit to eq 7.

determined from the relation

$$D = d^2/4\pi^2\tau \quad (8)$$

where the diffusion coefficient $D$ is expressed in m$^2$ s$^{-1}$. A fluorescence recovery graph with the fitted curve, obtained with modeling by eq 7, is shown in Figure 3.

The values of the diffusion coefficient at several temperatures obtained with the concentration profile and the FRAP method are shown in Table 1. The diffusion coefficient measurements were not carried out exactly at the same temperature for the two techniques. But as the temperature could deviate from the set-point temperature of ±0.2 °C for FRAP and ±0.5 °C for concentration profile measurements, the results can be compared as they are presented. The higher the temperature or the lower the concentration (or the viscosity), the faster was the diffusion of fluorescein, as expected from the Stokes–Einstein relation

$$D = kT/6\pi\eta r \quad (9)$$

with $T$ the temperature in Kelvin, $k$ the Boltzmann constant, $\eta$ the viscosity of the matrix in Pa·s, and $r$ the hydrodynamic radius of the diffusant in m.

The data obtained with the two methods are identical even at subzero temperature with or without gel. Whereas gel is necessary to the concentration profile method, it is not essential to the FRAP method. Our results demonstrate that there was no significant difference between diffusion coefficient values of fluorescein in 57.5% sucrose solution measured with or without 1% agar–agar. The FRAP experiments confirm that the gel at the concentration of 1% in viscous sucrose solutions has no significant influence on the fluorescein diffusivity. The diffusion coefficient values for sucrose in gel with agar–agar concentration in a range between 0.5% and 2% were similar to those in water (Bettenfeld, 1985; Lebrun and Junter, 1993).

The diffusion of fluorescein in gel media has been well documented in the literature (Mustafa et al., 1993; Bu and Russo, 1994) and was selected as the diffusing substance in the two methods for several reasons. The molecule of fluorescein used as a small probe presents several analogies with the sucrose molecule (Table 2): it is similar in size and molar volume to sucrose, the major constituent of the matrix. Furthermore, the fluorescein, used as a tracer for diffusion measurements, is convenient since very small amounts are sufficient for the two techniques. Literature data on diffusion coefficients of fluorescein and sucrose at 25 °C and at infinite dilution in water confirm the idea that fluorescein is a relevant probe for our purpose.

The diffusion coefficient values obtained for a small molecule (like fluorescein) can be influenced by the method of determination (Loncin, 1976). All of the fluorescein diffusion coefficient values, shown in Table 2, were determined by FRAP and are similar despite the different authors; however, a value of $D_0 = 353\ 10^{-12}$ $m^2\ s^{-1}$ was reported by Häglund et al. (1988) for fluorescein in water. This value, very different from the values given in Table 2, was obtained with a versatile shear cell fitted essentially for liquid media. So it is very interesting to have two different methods to confirm the values obtained for the determination of a diffusion coefficient. The FRAP method and the concentration profile method, chosen for the diffusion coefficient determination of a small molecule in viscous media, even at subzero temperatures, strengthen the values obtained in our investigations.

**Conclusions.** We have shown that the concentration profile and the fluorescence recovery after photobleaching techniques can be applied for measuring the small diffusion coefficients that occur in concentrated carbohydrate solutions.

The data obtained with the two methods were similar at 20 °C and at subzero temperatures. There are a number of interesting features of the concentration profile and FRAP experiments that make them extremely useful in a wide range of molecular studies:

(1) Only very small dye amounts are required for each of them.

(2) The FRAP technique allows investigators to carry out an experiment more rapidly than the profile technique. It is necessary to let the tagged molecules diffuse over a macroscopic distance (0.5–3 cm) if accurate results are needed with the profile method, while the diffusion length for FRAP is equal to the fringe spacing (1–100 $\mu$m). For example, whereas 45 days was necessary with the concentration profile, only 3 h was required for the same experiment with the FRAP method.

(3) The main FRAP requirement is that the sample has to be optically transparent for a thickness of the order of 1 mm at least. At subzero temperature, some samples contained ice; in that case the FRAP measurement was not possible, while the concentration profile measurement could allow the diffusion coefficient determination.

The two techniques complement one another for diffusion measurement and allow experiments in very viscous media for a wide range of low temperatures, with or without ice in the sample.

The main objective of these studies is to understand the molecular mobility of a small molecule at a temperature around the matrix glass transition temperature ($T_g$). Frick (1989) reported values around $10^{-17}$ $m^2\ s^{-1}$ for the diffusion coefficient of a small synthetic molecule, Aberchrome, in polystyrene matrix at $T_g$; Zhang and Wang (1994) evaluated the diffusion coefficient of a small dye, camphorquinone, in a synthetic polymer, polysulfone, to be around $10^{-16}$ $m^2\ s^{-1}$ in the vicinity of $T_g$. These data are a rough estimate of the diffusion coefficient expected for our food model system at $T_g$. These values of diffusion coefficient can be measured by using the FRAP technique. So these measurements are useful, both to the understanding of practical problems about stability related to concentrated or low-temperature systems and to the theoretical treatment of translational diffusion of small molecules below the glass transtion temperature.

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