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The role of structural relaxation in governing the mobility of linoleic acid in condensed whey protein matrices

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ABSTRACT

The classical limiting case of simple diffusion as described by Fick's second law was examined in the transport of a small molecule, linoleic acid, through a condensed polymer matrix, whey protein. Experimental protocol was based on small-deformation dynamic oscillation in-shear, wide angle X-ray diffraction, Fourier transform infrared spectroscopy, scanning electron microscopy, FTIR micro-spectroscopy imaging, ANS fluorescence spectroscopy, and the sulfo-phospho-vanillin assay. This mass transfer problem for the omega-6 fatty acid was examined in relation to whey protein forming a glassy system with a glass transition temperature, T_g, of −16 °C. Diffusion followed a more complicated pattern than Fick's equation that could be described at temperatures above T_g with the so-called "anomalous transport". The diffusion coefficient of linoleic acid was estimated within the glass transition region and glassy state of the whey protein network delineated with changing environmental temperature. The free-volume theory of transport was then considered to provide a useful vehicle for rationalising molecular motion and, in doing so, we established a generalised relationship between diffusion coefficient of bioactive compound and fractional free volume of polymeric matrix.

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1. Introduction

The concept of diffusion that emerged from physical sciences, with a paradigmatic example being the heat induced Brownian motion, is of increasing interest in functional food and nutraceutical manufacturing for the delivery of biofunctionality (Korsmayer & Peppas, 1981). Orally ingested embodiments for health benefit or medicinal use are hardly stable or equilibrium systems yielding "surprising" changes in structural morphology and consistency during storage and consumption. Clearly, advanced-formulation engineering requires that the time dependence of the statistical distribution of bioactive compounds in the three dimensional lattice is accurately followed by a differential diffusion equation (Slade & Levine, 1991). Solutions to this problem should primarily deal with the rate of molecular transport, which is governed by the diffusivity in the surrounding environment and the concentration gradient between adjacent demixed phases.

Small-molecule diffusion is driven by a chemical potential difference in the interfacial area of flat surfaces or decrease in Gibbs free energy, as shown by the classical depiction of a molar free energy diagram where diffusant transport occurs along its concentration gradient (Chantawansri, Yeh, & Hsieh, 2015). It is widely recognised that Adolf Fick in 1855 was the first to elaborate mathematical descriptions through his first law that applies to steady state systems, i.e. where the concentration of diffusant molecules remains constant. A more relevant case in model biological materials and food preparations, however, is that of Fick's second law following concentration changes with time (Rahman, Al-Marhubi, & Al-Mahrouqi, 2007). This allows the description of diffusion kinetics as applied, for example, in a number of practical cases including homogenisation of lipids whose segmental mobility in a glassy bread matrix is temperature dependent (Roudaut, Van Dusschoten, Van As, Hemminga, & Le Maste, 1998).

Spin-offs of understanding the meaning and application of Fick's second include the estimation of activation energy (4–8 kcal/mol) for the diffusion of lipids through cell membranes, and their diffusion coefficient (10^{-8} – 10^{-11} cm^2/s) in the bulk or along several defects of the gel phase in processed foods (Derzko & Jacobson, 1980). More recently, however, it became apparent that small-molecule diffusion through a glassy polymer often cannot be rationalised on the basis of a concentration-dependent diffusion coefficient. This "anomalous effect" is readily observed once

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spheres, cylinders or slabs of a polymeric material are placed in contact with a solvent, with the ensuing sink-diffusion model exhibiting an upward curvature in the relationship of diffusant weight increase ($\Delta\omega$) *versus* $t^{1/2}$ (Singh & Chauhan, 2009). In contrast, classical Fickian kinetics argues for an initial proportionality between $\Delta\omega$ and $t^{1/2}$.

Today, anomalous transport is known to describe non-Fickian kinetics and commonly involves cross-linked polymers in the glass transition region. This limiting condition is designated as "Case II", as opposed to "Case I" for Fickian kinetics, and is characterised by a much higher bioactive compound mobility than the segmental relaxation rate, with the polymer relaxation becoming the rate determining step (Crank, 1975). Therefore, analysis of important mass transfer problems for the functional foods industry requires knowledge of the polymer network characteristics in relation to the temperature dependence of diffusion coefficient in polymer-solvent systems (Dissanayake et al., 2012).

Synthetic polymer research has considered the free-volume theory of transport as a useful expedient for describing the physical picture of polymer-network effects on molecular motion. Polymer relaxation creates expanded holes, which are filled by molecules or "jumping units" of a bioactive compound, and this fluctuation in the local free volume allows diffusion with the formation and disappearance of holes (Tramon, 2014). The present investigation examines the aforementioned conceptual approaches on a high-solid matrix of whey protein that is capable of creating structures to hold bioactive microconstituents (Hudson, Daubert, & Foegeding, 2000). Experimental data obtained for the diffusion of linoleic acid in the polymeric system will be used to carry out a critical discussion of the applicability and predictive capabilities of the combined free volume/molecular diffusion theory in this type of materials.

2. Materials and methods

2.1. Materials

2.1.1. Whey protein isolate

It was a microfiltration-isolated powder from Fonterra Co-operative Group Ltd (Palmerston North, New Zealand), and contained 90.4% protein (N x 6.38), 4.7% moisture with the minor addition of carbohydrate (0.9%), fat (1.0%) and minerals (3.0%). The material has been tested for microbiological contamination, which was <10 cfu/g for yeast and mould, and the aerobic plate count was <10,000 cfu/g. Physical tests showed bulk density of 0.34 g/ml for the powder and pH 6.9 of 5% (w/w) solution at 20 °C.

2.1.2. Linoleic acid (cis-9,cis-12-octadecadienoic acid)

The fatty acid was obtained from Sigma Aldrich Co (Sydney, Australia). It is a high purity material (≥98.5% by GC) with an average molecular weight of 280.45 g/mol and density of 0.902 g/ml at 25 °C.

2.1.3. Reagents

8-Anilino-1-naphthalenesulfonic acid ammonium salt (ANS), with more than 97.0% purity and HPLC grade, was purchased from Sigma Aldrich Co (Sydney, Australia). Analytical reagents for the phosphate buffer, i.e. potassium dihydrogen phosphate (≥99.5% purity) and disodium hydrogen phosphate anhydrous (99.0% purity) were purchased from BDH Chemicals Ltd, Poole, UK.

2.2. Methods

High-solid sample preparation: Whey protein dispersion was prepared by adding 30% powder in Millipore water at room temperature with constant stirring on a magnetic plate. The preparation was continuously stirred for 2 h until perfectly dispersed and then stored overnight at 4 °C for complete hydration and removal of air bubbles. Sample was removed from the refrigerator and stirred for another 15 min at ambient temperature before adding 1% linoleic acid. Mixing was extended for another 30 min for thorough dispersion using a conventional magnetic stirrer. The mixture was further homogenised for 3 min at 3000 rpm with a laboratory homogeniser (Ultra-Turrax T25, IKA-Labortechnik, Staufen, Germany). Preparation was then concentrated in a rotary evaporator at 40 °C to achieve a level of 80% (w/w) solids. Concentrated matrix of 80% whey protein excluding linoleic acid was also prepared as the standardised system.

2.3. Experimental analysis

2.3.1. Rheological measurements

Viscoelastic properties of condensed samples of 79% whey protein isolate with 1% linoleic acid (and 80% whey protein isolate) were analysed using small-deformation dynamic oscillation in shear with the Advanced Rheometer Generation 2 (AR-G2 from TA Instruments, New Castle, DE) equipped with magnetic trust bearing technology. Samples were loaded onto the Peltier plate of the instrument at 25 °C with a set gap of 1000 μm and a 10 mm parallel-plate measuring geometry. Sample edges were covered with silicone oil (BDH, 50 cS) to minimize moisture loss. To monitor changes in storage (G') and loss (G'') modulus with temperature, a constant scan rate of 1 °C/min, oscillatory frequency of 1 rad/s and strain of 0.01%, which was tested to be within the linear viscoelastic region (LVR), were applied throughout the experimental routine (maintaining a normal force of 0.08 N throughout).

This controlled strain rheometer was connected to a 60 L liquid nitrogen tank to provide purging nitrogen gas, which, in this work, cooled the samples down to −38 °C. Frequency-sweep data were collected from the lowest experimental temperature to 6 °C within a range of angular frequencies of 0.1–100 rad/s at constant temperature interval of four degrees centigrade. Storage and loss modulus were plotted against reduced angular frequency data to obtain the master (or composite) curve of viscoelasticity. The principle of time-temperature superposition (TTS) was then applied to estimate the so-called mechanical glass transition temperature (T_g) using appropriate modeling.

2.3.2. Fourier transform infrared spectroscopy (FTIR)

The technique was employed to identify potential alteration in chemical fingerprints as a result of adding fatty acid to the high-solid system. Samples of 79% whey protein with 1% linoleic acid were thus prepared along with single preparations of 80% whey protein and linoleic acid, which served as the standard. Work was performed using a Perkin Elmer Spectrum 100 with MIRacle™ZnSe single reflection ATRplate (Perkin Elmer, Norwalk, CT). All materials were scanned within the range of 600–4000 cm^{-1} with a resolution of 4 cm^{-1} averaged over thirty two scans, and each experimental sample was analysed in triplicate.

2.3.3. Wide angle X-ray diffraction (WAXD)

Bulk structure of 79% whey protein with 1% incorporated linoleic acid in comparison with whey protein powder and 80% high-solid protein preparation was examined using a Bruker D4 Endeavour (Karlsruhe, Germany). Freeze-dried samples were loaded onto the X-ray measuring compartment, scanned at the accelerating voltage of 40 kV and current of 40 mA using a position sensitive detector (PSD) within the 2θ range of 5–90° in measuring intervals of 0.1°. Data were converted with DIFFRAC^{plus} Evaluation (Eva), version 10.0, revision 1 and each experimental sample was

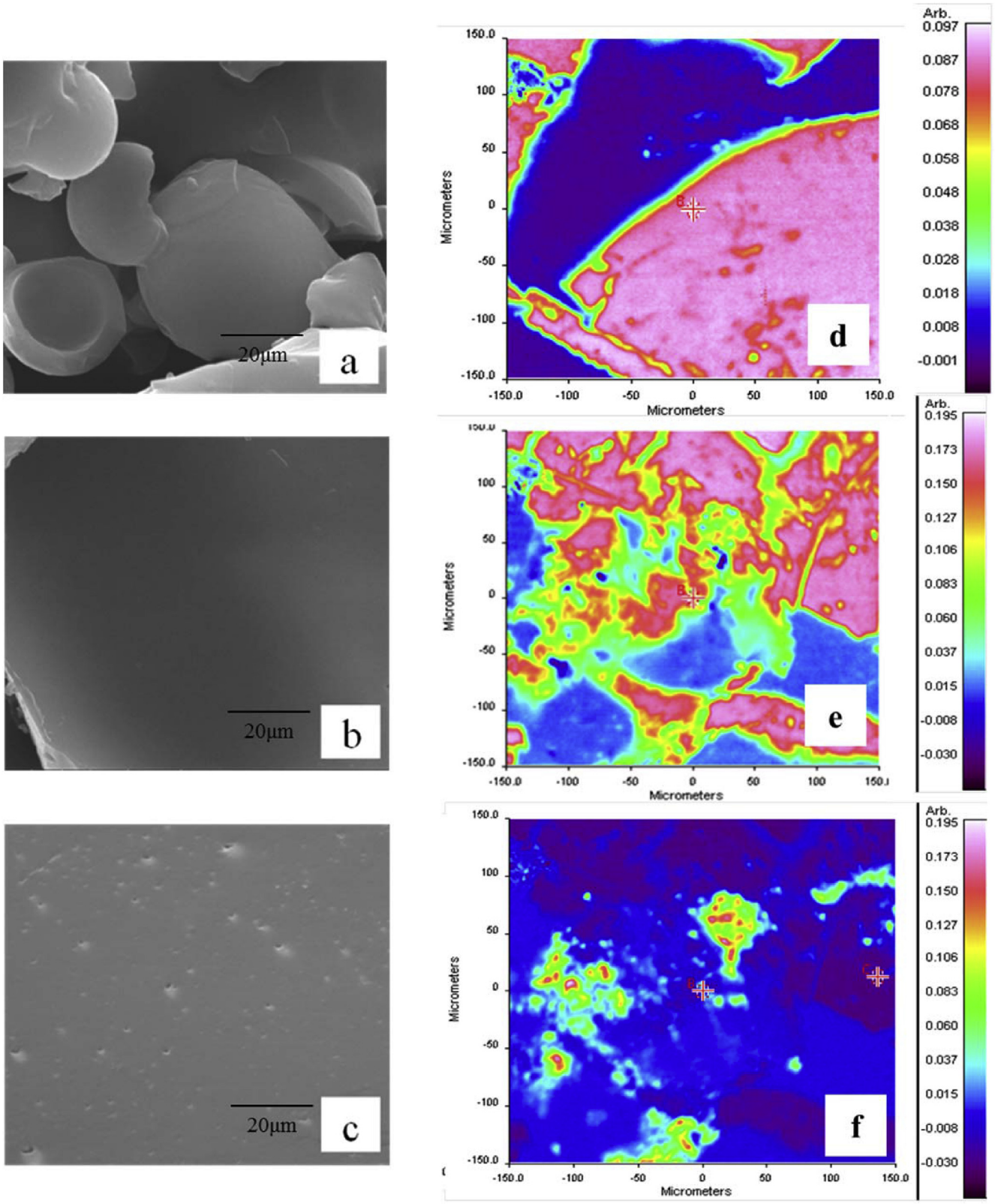

Fig. 5. SEM micrographs for (a) native whey protein powder, (b) 80% whey protein, (c) 79% whey protein with 1% linoleic acid, and FTIR microscopy imaging for (d) 80% whey protein to identify the protein distribution within 1700–1500 cm^{-1}, (e) 79% whey protein with 1% linoleic acid for protein distribution within 1700–1500 cm^{-1} and (f) 79% whey protein with 1% linoleic acid for linoleic acid distribution within 1470–1350 cm^{-1}.

amount of lipid released within the present experimental settings.

Diffusion exhibits a linear increase up to 10 h of observation, and an extra dimension of this process was obtained by plotting data as a function of experimental temperature from −30 to 10 °C in

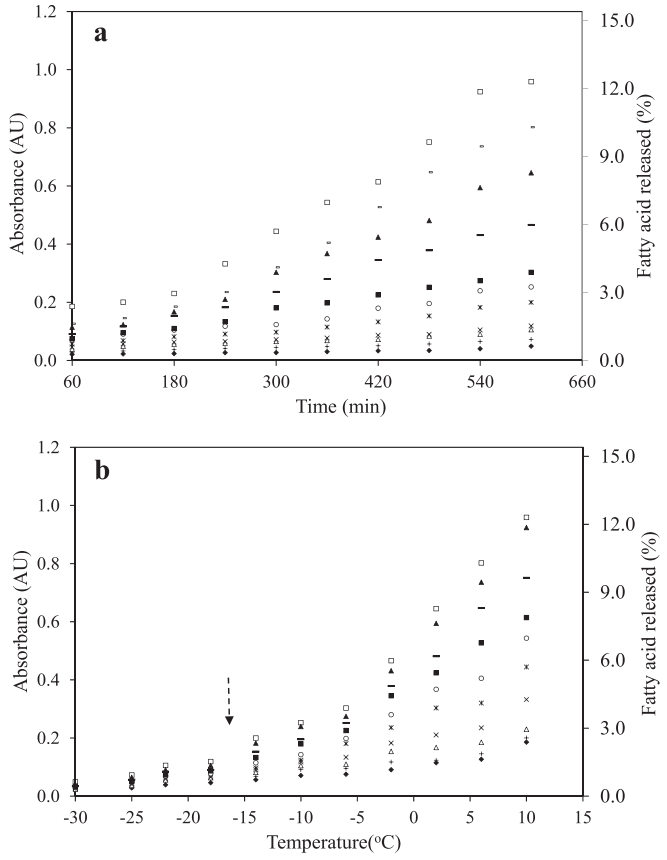

Fig. 6. Absorbance and percentage release of 1% linoleic acid diffused through 79% whey protein (a) as a function of time of observation at −30 (◆), −26 (+), −22 (Δ), −18 (x), −14 (⋆), −10 (○), −6 (■), −2 (⁻), 2 (▲), 6 (−), 10 (□), and (b) as a function of temperature of observation at 60 (◆), 120 (+), 180 (Δ), 240 (x), 300 (⋆), 360 (○), 420 (■), 480 (⁻), 540 (▲), 600 (□) min obtained at 525 nm (arrow indicates the mechanical T_g).

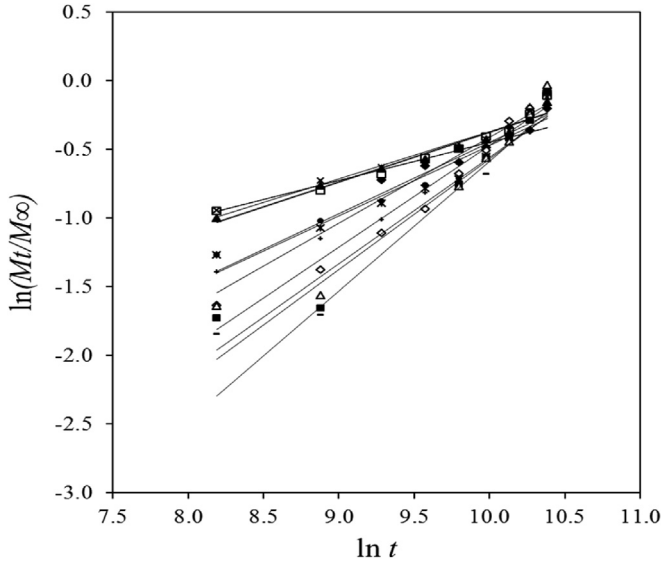

Fig. 7. Absorbance in $ln(M_t/M_\infty)$ as a function of time of observation ($ln\ t$) at −30 (◆), −26 (□), −22 (▲), −18 (x), −14 (⋆), −10 (●), −6 (+), −2 (◊), 2 (■), 6 (−), 10 (Δ)° C for 79% whey protein with 1% linoleic acid.

Fig. 6b. Statistical significance was identified for each level of observation time and the experimental temperature ($p < 0.05$ from Tukey's post hoc analysis). It is clear that the rate and amount released drops dramatically at temperatures below T_g (−16 °C), as pinpointed by thermomechanical work. We have taken advantage of the good linear relationship between absorbance and time monitored for all temperatures tested ($r^2 = 0.980 \pm 0.010$) to argue for a zero-order reaction rate, with the gradient being the rate constant, $k = dx/dt$. This can be utilised to calculate the so-called "spectroscopic shift factor" for our temperature range, as follows (Kasapis & Shrinivas, 2010):

$$\log a_T = \log \frac{k_o}{k} \tag{4}$$

where, k_o is the rate constant at the reference temperature, $T_o = -14$ °C.

Spectroscopic shift factors for the diffusion of linoleic acid in a condensed whey protein matrix have also been plotted in Fig. 2 using the modified Arrhenius equation. This produces a high-quality linear fit ($r^2 = 0.990$) throughout the experimental temperature range of interest that contrasts strongly with the pattern of polymer structural relaxation. Moreover, the activation energy is computed to be 55 kJ/mol, i.e. much lower that for whey protein (quoted earlier at 335 kJ/mol), an outcome which argues that the fractional free volume of diffusion for the microconstituent is larger than the fractional free volume of viscoelastic relaxation in the whey protein network.

The gist of modelling thermomechanical and spectroscopic data in Fig. 2 is that although whey protein controls the diffusion of linoleic acid, the physics of the two molecular processes, i.e. structural relaxation of the matrix and diffusion kinetics of the bioactive compound, are distinct. Analysis of such molecular phenomena is further facilitated if a reliable theory is available for describing the temperature dependence of the diffusion coefficient of the fatty acid. In doing so, we considered the widely used but empirical power law equation that provides the kinetic diffusion exponent, n, as follows:

$$\frac{M_t}{M_\infty} = kt^n \tag{5}$$

where, M_t/M_∞ is the cumulative amount of bioactive compound release at experimental and equilibrium time, k is a constant characteristic of the bioactive compound-polymer system, and t is given in seconds.

Absorbance data from Fig. 6a were modelled for 10 h to produce relevant parameters from the slope and intercept in the plot of $ln\ M_t/M_\infty$ *versus* $ln\ t$ (Fig. 7 and Table 1). Ideal Fickian kinetic (Case I) is reflected in an n-value of 0.5, whereas Case II transport is characterised by an n-value of 1.0, which makes the range of n-values between 0.5 and 1.0 a Non-Fickian or anomalous diffusion (Bajpai, Bajpai, & Shukla, 2001; Wang, Wu, & Lin, 2008). Values of the diffusion exponent in this investigation were found to lie between 0.28 and 0.85 with increasing temperature. They suggest a Less-Fickian behaviour in the glassy state turning to anomalous transport above the glass transition temperature. In the former, lipid transport is the rate-limiting process but temperature increase leading to polymer plasticisation means that the anomalous lipid diffusion occurs simultaneously with the structural relaxation of the monolithic matrix (Masaro & Zhu, 1999).

The above school of thought can be further utilised to estimate the diffusion coefficient, D_{eff}, of linoleic acid from the condensed matrix of whey protein to the external collection tank of ethyl acetate, due to a concentration-gradient differential, using the following mathematical expression (Wang et al., 2008):

Table 1
Diffusion exponent (n) and system characteristic constant (k) calculated using power law equation for linoleic acid entrapped in condensed whey protein.

Temperature (°C)	Diffusion exponent n	System characteristic constant k	Diffusion mechanism
−30	0.28	6.24E-04	Less-Fickian
−25	0.36	1.05E-04	Less-Fickian
−22	0.36	1.00E-04	Less-Fickian
−18	0.34	1.63E-04	Less-Fickian
−14	0.51	2.62E-05	Anomalous transport
−10	0.52	2.40E-05	Anomalous transport
−6	0.62	2.26E-07	Anomalous transport
−2	0.74	1.35E-08	Anomalous transport
2	0.80	2.50E-09	Anomalous transport
6	0.85	6.63E-10	Anomalous transport
10	0.77	4.95E-09	Anomalous transport

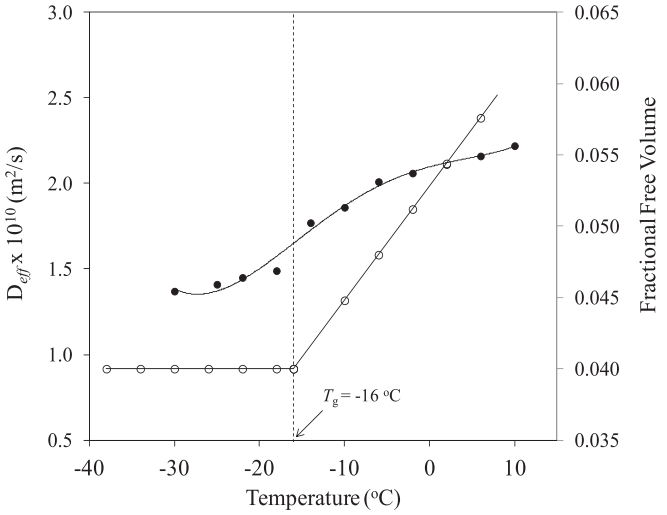

Fig. 8. Diffusion coefficient (D_{eff}) of 1% linoleic acid through 79% whey protein within 10 h (●, left y-axis) and fractional free volume (f_0) of the condensed matrix within the glass transition region and glassy state (○, right y-axis).

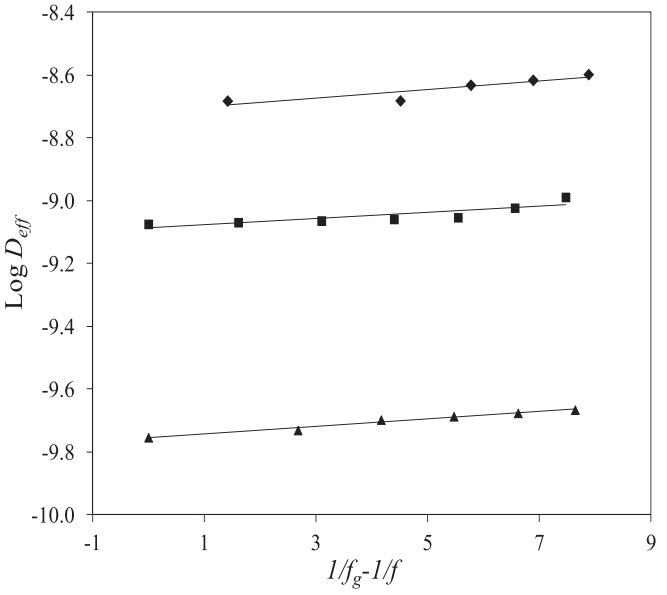

Fig. 9. Graphic of diffusion coefficient against an inverse function of fractional free volume at $T \geq T_g$ for oleic acid (◆), α-linolenic acid (■), and linoleic acid (▲).

$$\frac{M_\infty - M_t}{M_\infty - M_i} = 4\left(\frac{D_{eff}\, t}{\pi L^2}\right)^{1/2} \tag{6}$$

where, M_i, M_t, and M_∞ denote the absolute amounts of the diffusant compound released at times zero, during experimentation and infinity/equilibrium, respectively, and L is the thickness of the slab.

Equation (6) has been used to model successfully Less-Fickian kinetics in the oral delivery of insulin encapsulated with psyllium polysaccharides (Singh & Chauhan, 2009), and achieved very acceptable data fitting in this investigation as well ($r^2 = 0.920$). It yielded in Fig. 8 a set of values for the diffusion coefficient spanning the range around the glass transition temperature. There is considerable variation in the diffusion-coefficient predictions from 2.30×10^{-10} to 1.37×10^{-10} m^2/s over the experimental range of forty degree centigrade. We also show in this juncture the values of fractional free volume for the polymeric matrix, which descend from 0.060 to 0.040 in the passage from the glass transition region to the glassy state.

Profiles of diffusion coefficient and fractional free volume in Fig. 8 indicate that there is a fundamental relationship between the two theoretical parameters at $T \geq T_g$. This type of quantitative relationship has been advanced earlier for the transport of water soluble vitamins through the condensed matrix of hydrocolloids in the form of slabs or spheres (Panyoyai & Kasapis, 2016). It proposed the following equation that develops a workable protocol for the free volume theory of diffusion:

$$\log\left[\frac{D_{eff}(T)}{D_{eff}(T_g)}\right] = \frac{\xi}{2.303}\left[\frac{1}{f_g} - \frac{1}{f}\right] \tag{7}$$

where, ξ represents the critical molecular volume of the jumping unit of a bioactive compound to that of the polymer matrix.

Application of Equation (7) to our data generates a linear relationship between diffusion coefficient and the inverse of fractional free volume in Fig. 9. We also plot for the first time corresponding data for the transport of 1% α-linolenic acid through 2% κ-carrageenan (50 mM K^+; pH 4.5) plus 82% polydextrose slabs, and 1% oleic acid through 3% high-methoxy pectin (pH 3) plus 81% glucose syrup slabs (Paramita, Bannikova, & Kasapis, 2015; Paramita, Bannikova, & Kasapis, 2016). In all cases, a linear relationship was obtained, as proposed by Equation (7), for log D_{eff} *versus* $(1/f_g - 1/f)$ thus being encouraging for its adoption in hydrocolloid based mixtures with a bioactive compound.

In light of the molecular coupling theory (MCT), ξ is essentially a coupling parameter whose value is a function of the nontrivial interactions between bioactive compound and hydrocolloid matrix (Ehlich & Silescu, 1990; Guo, Knight, & Mather, 2009). Therefore, the term 1 - ξ becomes a fundamental concept that describes the level of decoupling between fatty acid diffusion and hydrocolloid

structural relaxation. Diffusion coefficient-fractional free volume fits for all materials presented in Fig. 9 produce decoupling values of 0.995, whereas these were about 0.70 for strongly interacting hybrid polyurethanes in biodegradable stent coating (Guo et al., 2009). The high values of decoupling reported for hydrocolloid-fatty acid blends are congruent with modeling in Fig. 2, which also argues for reduced cooperativity between structural relaxation of polymeric network and diffusion of microconstituent.

4. Conclusions

The present investigation considered several attributes of the free-volume theory to study the vitrification of a condensed whey protein matrix in the presence or absence of linoleic acid. Modeling indicates a break in the magnitude of mobility in the two constituents with environmental temperature due to excess free volume characterising the transport of fatty acid in the glassy state. Predictions developed to describe diffusivity *versus* temperature relationships make the glass transition temperature a parameter of physical significance that alters diffusion characteristics from Less-Fickian to anomalous transport in accordance with the thermally-induced polymer plasticisation. Examination of the glassy polymer at variable temperatures unveils a qualitative agreement between free volume variation and diffusion of fatty acid. This allows the description of a coupled relaxation-diffusion mechanism, with experimental measurements being able to consistently identify the level of molecular interaction between macromolecule and bioactive compound in these mixtures. Common examples of model food systems have been given here and earlier (fatty acids or vitamins), and the generic nature of this approach could be used to describe further types of mass transfer phenomena available in the literature.

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