An Assay of Molecular Mobility in Solid Corn Meal by Front-Face Fluorescence Anisotropy

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ABSTRACT

A new technique based on front-face fluorescence anisotropy was developed to study molecular mobilities in solid corn meal samples before and after extrusion cooking. Anisotropy was determined by measuring the polarization of fluorescence of vitamin A, a natural constituent of corn meal, or that of an added probe, calcein. A conventional fluorescence spectrophotometer was modified by constructing a cuvette holder that allowed the cuvette, filled with a powdered sample, to be oriented at variable angles. Positive anisotropy errors due to polarized stray light were measured and allowed for. Negative errors due to depolarization at air-sample interfaces were eliminated by immersing the powdered samples in a liquid of matching refractive index. In uncooked corn meal the anisotropies were low, indicating high mobility of the fluorophore. In corn meal extrudates, the anisotropies increased with increasing extrusion temperature and decreasing moisture, showing loss of mobility. Anisotropy measurement is a simple and rapid method for relating structural changes at the molecular level with process conditions.

Excitation of a fluorescent molecule by plane-polarized light causes the emitted fluorescence also to be polarized. If the molecule undergoes rotational motion while in the excited state, then the plane of polarization of the emitted light will change direction, resulting in depolarization of the fluorescence. As shown in standard texts (e.g., Lakowicz 1983), the extent of depolarization is a measure of the rate of rotational motion of the fluorophore. In the case of polymeric networks, as in corn meal, such motion can be interpreted as flexibility of the polysaccharide segments. Results are commonly expressed as fluorescence anisotropy, $\bar{r}$, as a measure of structural rigidity.

The molecular mobility of systems such as polymers, surface films, etc., must be studied by examining the samples in the solid state, since any solution process destroys the very bonds between molecules or segments one wishes to characterize. We here describe a simple assay for mobility, using front-face anisotropy measurements. The method was developed in the course of studying the starch-protein-lipid matrix of corn meal before and after it was subjected to heat and shear in an extrusion-cooking process. Front-face anisotropy was found to be a sensitive parameter of molecular rigidity that correlated well with bulk physical properties of the samples such as bulk density, compressive strength, and puff ratio (expansion of the extrudate as it emerges from the extruder die). This technique has so far been described only for liquid samples (Blumberg et al 1977, Eisinger and Flores 1979, Winkelman and Grossman 1967), although there are brief references to its use with polymer films (Nishijima 1970).

MATERIALS AND METHODS

Samples of corn meal or corn flour were used without further processing. Extrusion-cooked samples were either chopped by hand with a razor blade into approximately 1-mm pieces or passed through a Wiley cutting mill (Arthur H. Thomas Co.), producing 20- or 40-mesh powders. Powdered samples were measured in a 10 × 10 mm quartz cuvette. They were analyzed either in the dry state, or alternatively were immersed in *n*-decane (Fisher Scientific Co.) and then deaerated under vacuum. The second procedure was used to eliminate errors due to depolarization at the numerous sample-air interfaces in powdered samples.

In other experiments, corn flour was mixed with water to form a thick paste that was spread in a thin layer on glass slides. Samples were dried either at room temperature or by heating in an oven at 210°C for 20 min while covered by another glass slide in order to slow down the drying rate and thus to subject the sample to heat and moisture. In another set of experiments, calcein (bis-*N*,*N*-biscarboxymethylaminomethyl-fluorescein, from Aldrich Chemical Co.) was added as a dilute aqueous solution to a corn meal-water paste before drying in either of the above ways. The glass slides carrying dried corn meal films were mounted vertically in the fluorescence spectrometer for anisotropy measurement.

A special holder was constructed to position the cuvette off-center in the sample compartment of a Hitachi-Perkin Elmer MPF-3 fluorescence spectrophotometer. As shown in Figure 1, the cuvette containing a powdered sample could be rotated and also moved along a diagonal slide. These adjustments permitted the excitation beam to be focused on a point near the inside of the cuvette face at any desired angle of incidence. Alternatively, a glass slide carrying a sample film could be clamped vertically at variable angles.

Polarizing filters for the near-ultraviolet and visible spectral range were placed in the excitation and emission beams of the spectrometer. Fluorescence intensities were measured first with both polarizers in the vertical position (I_{vv}), then with the emission

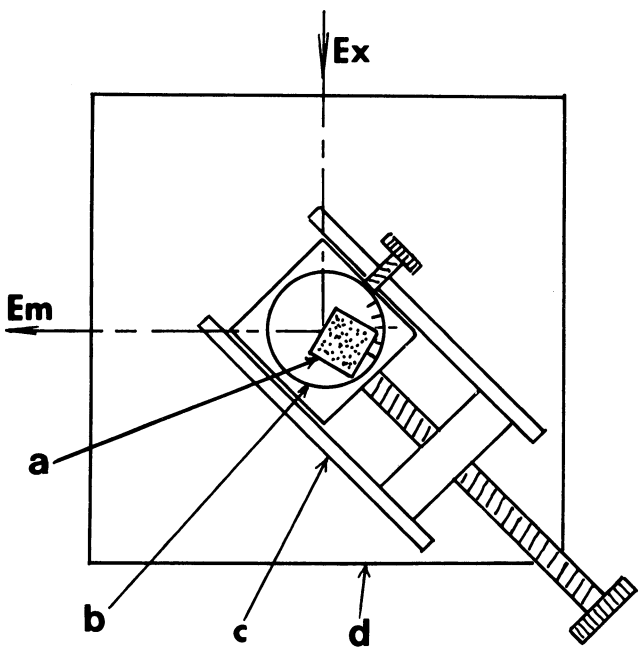

Fig. 1. Cuvette holder for front-face fluorescence anisotropy measurements. Directions of the excitation and emission beams are indicated by **Ex** and **Em**. A 10 × 10 mm quartz cuvette containing a powdered sample (**a**); rotatable cuvette holder with set screw and angular marks (**b**). The cuvette face passes through the center of rotation. Slide and screw for diagonal positioning of the cuvette (**c**). Platform fitting into the sample compartment of the fluorescence spectrophotometer (**d**).

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polarizer turned to the horizontal position (I_{vh}). To correct for any polarization caused by the monochromators of the instrument, a correction factor G, defined as I_{hv}/I_{hh}, was determined for each sample by appropriate orientation of the polarizers. The anisotropy, $\bar{r}$ was calculated by the expression

$$\bar{r} = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh}) \quad (1)$$

(Lakowicz 1983). A high $\bar{r}$ value indicated restricted mobility of the fluorophore (high rigidity), and vice versa.

RESULTS

Fluorescence intensity maxima in solid corn meal were observed at two absorption-emission wavelength pairs of 296/355 and 350/450 nm. These were tentatively assigned to vitamin E and vitamin A, respectively, by comparing observed absorption and emission spectra with literature data (Sadtler 1974, Weber 1987, Wendler et al 1950). The more intense 350/450 nm peak due to vitamin A was chosen for further study.

Anisotropy values for powdered corn meal, corn flour, and an extrudate are given in Table I. The results show that the particle size had little or no effect on the $\bar{r}$ values. As shown in Table I, anisotropies were very low in uncooked samples but were considerably higher in extruded products.

A systematic variation of the angle of incidence on the cuvette face showed a large increase in $\bar{r}$ when the angle passed through 45°, evidently due to specular reflection (Fig. 2). All measurements therefore were made with the exciting beam at 30° to the cuvette face where slight variations in the angle had no effect.

There are two potential sources of error in anisotropy measurements on solid samples: 1) Light reflected from the cuvette face is vertically polarized. It is detected together with the fluorescence emission, causing apparent anisotropies to be too high. 2) Multiple scattering of both the incoming excitation and the outgoing emission at the sample-air interfaces of the powder particles causes some depolarization and causes apparent anisotropies to be too low.

Reflected light intensities were measured by placing nonfluorescent materials into the sample cuvette. Materials found suitable included Teflon tape or powder, glucose, sodium silicate, and sodium chloride, all of which gave comparable results. An emission spectrum, with excitation at 350 nm is shown in Figure 3A, curve 1. Stray light at 450 nm amounted to 0.5% of the intensity at 350 nm. The emission spectrum for corn meal, also with excitation at 350 nm, is shown in curve 2, where the emission peak at 450 nm represents the fluorescence plus any stray light. If we assume that the ratio of reflected light intensity at 450 nm to that at 350 nm is the same as in the nonfluorescent blank, 0.005, then the stray light intensity for corn meal is 0.005 × 20 or 0.10. This represents 5.5% of the total emission intensity of 1.8 at 450 nm.

A further check was made by labeling corn meal with calcein (Methods). This fluorophore has excitation and emission maxima at 468 and 520 nm that do not interfere with those of vitamin A. An emission spectrum of unlabeled corn meal, excited at 468 nm, is shown in Figure 3B, curve 3, where the emission intensity at 520 nm represents stray light only. Here the ratio of intensities at 520 and 468 was 0.0012. Emission spectra of calcein-labeled corn meal samples are shown in curve 4 for an unheated sample and in curve 5 for a sample after heating at 200°C for 20 min. Taking the stray light intensities at 520 and 468 nm to be the same as in the calcein-free control, we find the stray light values at 520 nm to be 0.066 and 0.054, representing 16 and 6.5% of the total intensity, respectively, for these two samples.

Stray light is vertically polarized, and thus affects the value of I_{vv} rather than that of I_{vh} in equation 1. A simple calculation shows that a 10% reduction in I_{vv} results in reduction in the

TABLE I
Effect of Particle Size on Fluorescence Anisotropy, $\bar{r}$

Sample	$\bar{r}$ (350/450 nm)
Uncooked corn meal, 30-mesh	0.031
Uncooked corn flour, 60-mesh	0.034
Corn meal extrudate, hand-chopped	0.129
Cron meal extrudate, 20-mesh	0.129

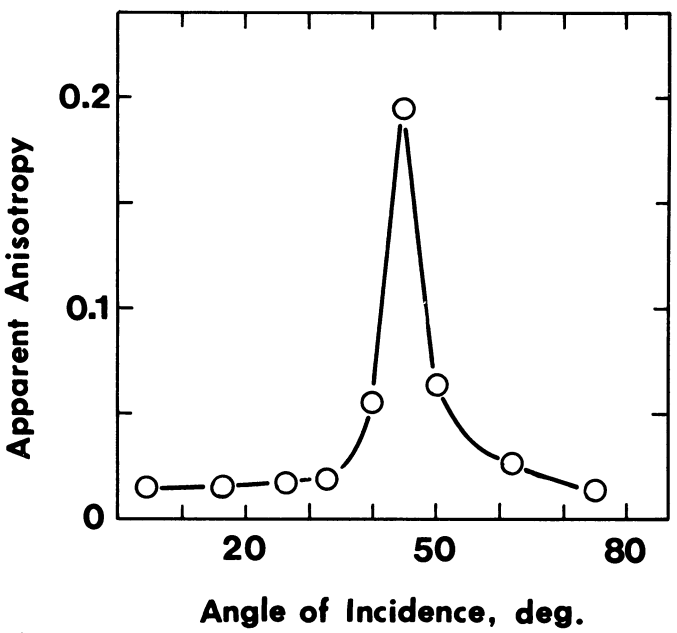

Fig. 2. Apparent fluorescent anisotropy as a function of the angle of incidence of the excitation beam on the cuvette face.

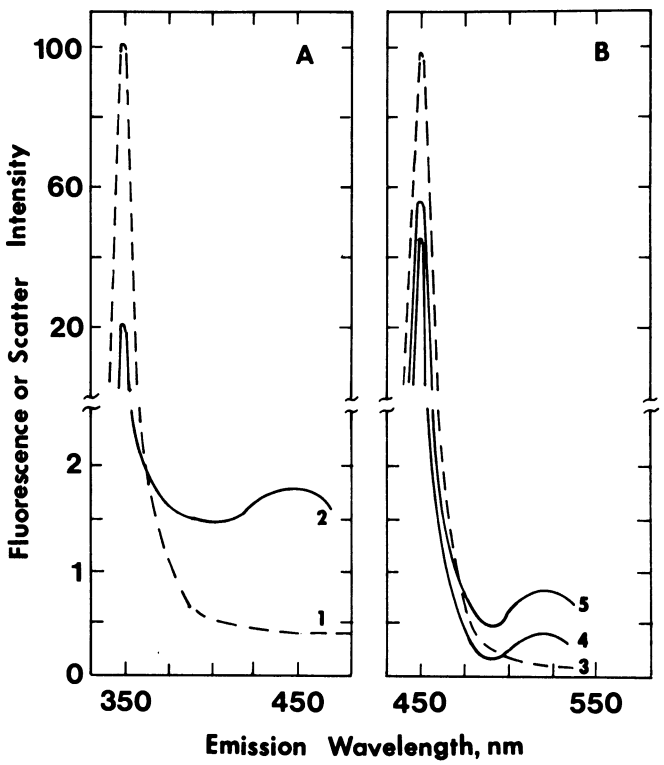

Fig. 3. Corrections of fluorescence intensities for stray light. **A**, Emission spectra of a nonfluorescent standard (sodium silicate, Teflon powder, or sucrose) (curve **1**) and of corn flour (curve **2**) when excited at 350 nm. The fluorescent component of corn flour is vitamin A, with absorption and emission maxima at 350 and 450 nm, respectively. **B**, Emission spectra of corn flour (curve **3**), corn flour labeled with calcein (curve **4**), and corn flour with calcein after heating at 200°C for 20 min (curve **5**). Excitation was at 450 nm.

absolute value of $\bar{r}$ by an amount that gradually increases from 0.03 to 0.04 as the anisotropy rises from 0.05 to 0.25.

The second source of error, depolarization at the many interfaces between sample particles and air, was eliminated, as far as possible, by immersing the samples in *n*-decane, chosen as a medium with refractive index close to that of carbohydrates. Application of a slight vacuum caused the decane to fill all voids. Four samples, uncooked corn meal and three extrudates, were measured for anisotropy in the dry state and in decane. Results are plotted in Figure 4. Values of $\bar{r}$(decane) were higher than $\bar{r}$(dry). The plot of these values against each other was linear but the line did not pass through the origin. This suggested that the depolarization error, causing an enhancement in $\bar{r}$(decane), is proportional to the magnitude of $\bar{r}$, but that both $\bar{r}$(decane) and $\bar{r}$(dry) must be corrected downwards by the error due to stray light, assumed to be constant.

This model is represented by

$$[\bar{r}(\text{decane}) - S] = D\,[\bar{r}(\text{dry}) - S] \qquad (2)$$

where $\bar{r}$(decane) and $\bar{r}$(dry) are observed values, S is the stray light error, and D is the enhancement factor due to elimination of the depolarization error. Solving this equation with the data from the above four samples gave $D = 2.55 \pm 0.03$, and $S = 0.04 \pm 0.03$. This result for S from direct anisotropy measurements is in excellent agreement with the error deduced (with certain assumptions) from stray light measurements. A plot of ($\bar{r}$[decane] − 0.04) versus [($\bar{r}$[dry] − 0.04) (x in Fig. 4) is seen to be linear and passing through the origin. Anisotropies corrected for both polarized stray light and for interfacial depolarization, $\bar{r}$(corrected), therefore are given by

$$\bar{r}(\text{corrected}) = \bar{r}(\text{decane}) - 0.04 \qquad (3)$$

or by

$$\bar{r}(\text{corrected}) = 2.55 \times (\bar{r}[\text{dry}] - 0.04) \qquad (4)$$

It is possible, therefore, to obtain corrected anisotropies from measurements on dry samples, or, possibly more accurately, from samples measured in decane. The values of S and D used for correction obviously need to be determined for each individual instrumental setup.

Fluorescence anisotropies both of the intrinsic vitamin A and of the added calcein fluorophore could be determined in the same sample since different wavelengths were involved. Data are given in Table II. Both fluorophores showed consistent trends, i.e., low anisotropies in samples air-dried at room temperature, and much higher anisotropies in heated samples. The anisotropies for vitamin A were higher than those of calcein by a factor of 3.

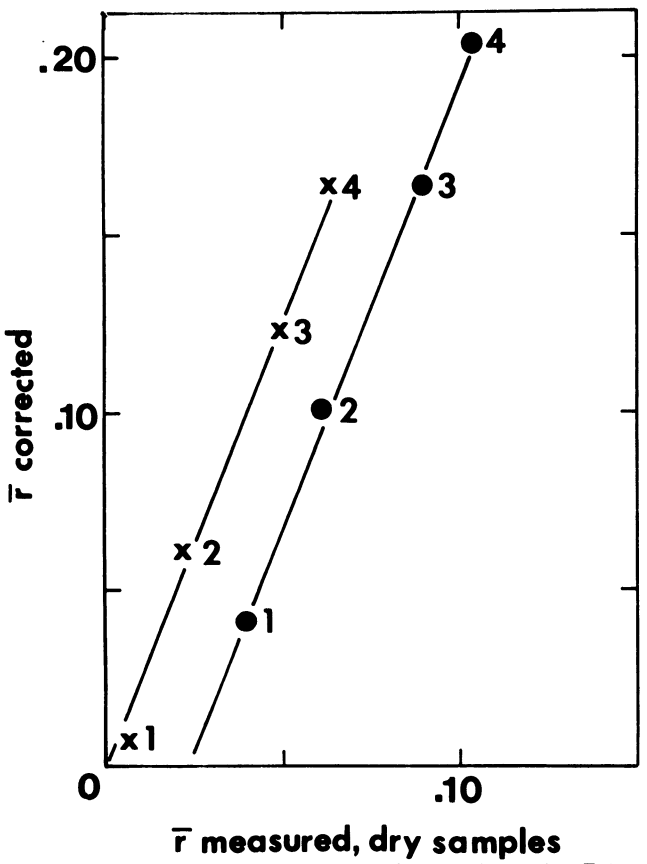

Fig. 4. Measured anisotropies of decane-immersed samples, $\bar{r}$(decane) (●), i.e., $\bar{r}$ corrected for interfacial depolarization only, as a function of anisotropies of the same samples measured in the dry state, $\bar{r}$(dry). Anisotropies corrected both for presence of polarized stray light and for interfacial depolarization, $\bar{r}$(corrected) (**x**), as a function of $\bar{r}$(dry) corrected for stray light. Numbers denote samples: **1** = uncooked corn meal, and **2**, **3**, and **4** = corn meal extruded at 200°C and with 30, 25, and 20% moisture, respectively.

DISCUSSION

The results presented here demonstrate that fluorescence anisotropies can be measured reproducibly in corn meal and extrudates. Measurements on solid samples involve errors due to polarized stray light and interfacial depolarization that normally do not occur with liquid samples. However, these errors can be eliminated by measuring samples when immersed in a liquid of matching refractive index and by other calibration procedures. The presence of a natural fluorophore in corn, and the lack of sensitivity of this method to particle size, means that samples can be analyzed without intrusive processing. The ability to correct for inherent errors by an equation or calibration curve means that samples can be tested routinely in the dry state. This opens the possibility of adapting this method to automation and on-line analysis.

A limited range of samples examined so far has shown a consistent correlation of observed anisotropies with processing conditions. In uncooked corn meal $\bar{r}$ was low, indicating that the fluorophore, and by implication also the starch-protein matrix, was relatively mobile. Heating of corn meal in the presence of moisture ranging from 8 to 25%, or passage through a screw extruder, i.e., subjection to heat and shear under these same moisture conditions, caused substantial increases in anisotropy, showing a loss in mobility. The magnitude of these increases could be correlated with temperature and moisture conditions during extrusion. For corn meal extrudates, the anisotropy was found to increase with increasing temperature and decreasing moisture, i.e., with increasing "harshness" of extrusion conditions. The anisotropy of extrudates also correlated well with physical and sensory properties such as compressive strength and sensory taste rating. Molecular mobility thus is shown to be a sensitive measure of various overall properties of extrudates and other processed cereal products. The anisotropy method is convenient for quality control and can be used to monitor changes in such products during long-term storage, such as staling due to starch retrogradation.

TABLE II
Comparative Anisotropies of Intrinsic and External Fluorophores

Sample	Intrinsic (Vitamin A) $\bar{r}$ (350/450)	External (Calcein) $\bar{r}$ (468/520)
Corn flour + calcein (room temperature)	0.037	0.012
Corn flour + calcein (210°C, 20 min)	0.199	0.067

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LITERATURE CITED

BLUMBERG, W. E., EISINGER, J., LAMOLA, A. A., and ZUCKERMAN, D. M. 1977. Zinc protoporphyrin level in blood determined by a portable hematofluorometer. J. Lab. Clin. Med. 89:712-723.

EISINGER, J., and FLORES, J. 1979. Front-face fluorometry of liquid samples. Anal. Biochem. 94:15-21.

LAKOWICZ, J. R. 1983. Fluorescence polarization. In: Principles of Fluorescence Spectroscopy. Plenum Press: New York.

NISHIJIMA, Y. 1970. Fluorescence methods in polymer science. J. Polym. Sci. Polym. Symp. 31:353-373.

SADTLER RESEARCH LABORATORIES INC. 1974. Pharmaceuticals Fluorescence Spectra 1:R103.

WEBER, E. J. 1987 Lipids of the kernel. Pages 311-349 in: Corn Chemistry and Technology. S. A. Watson and P. E. Ramstad, eds. Am. Assoc. Cereal Chem.: St. Paul.

WENDLER, N. L., ROSENBLUM, C., and TISHLER, M. 1950. The oxidation of carotene. J. Am. Chem. Soc. 72:234-239.

WINKELMAN, J., and GROSSMAN, J. 1967. Quantitative fluorescence analysis in opaque suspensions using front face optics. Anal. Chem. 39:1007-1009.

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