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Quantitative microscopy in carbohydrate analysis

R.G. Fulcher[a], J.M. Faubion[b], R. Ruan[c] & S. S. Miller[d]

[a] *Department of Food Science and Nutrition, University of Minnesota, St Paul, MN 55108, USA*
[b] *Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, USA*
[c] *Department of Agricultural Engineering, University of Minnesota, St. Paul, MN 551084, USA*
[d] *Centre for Food and Animal Research, Agriculture Canada, Ottawa, Ontario, Canada K1A 0C6*

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INTRODUCTION

> "The methods of photometry which Ehrlich could obtain
> Were nowhere near a match for one good eyeball and brain
> But cytophotometric apparatus, nowadays,
> Can help us class the *carbohydrates* in many different ways"

In modifying Shapiro's original quotation (Shapiro, 1977), we emphasize the utility of quantitative microscopy and histochemistry in carbohydrate analysis, an approach we have been enjoying for several years in a number of collaborative studies (e.g. Wood & Fulcher, 1983, 1984; Fulcher *et al.*, 1984; Miller *et al.*, 1984). A thorough review of many of the relevant techniques and applications is available elsewhere (Fulcher *et al.*, 1989). These techniques, including fluorescence, bright-field and near infrared (NIR) microscopy, microscope photometry (MP) and digital image analysis (DIA) are particularly useful in assessing the relationship between structure and function in biological materials in general, and carbohydrates in particular. In combination, these various techniques allow *in situ* identification and/or characterization of individual molecular species, and quantitation of these materials in cells, tissues, and food products.

PROCEDURES AND INSTRUMENTATION

Microscopy

Although the localization of cellular compounds using histochemical techniques is many decades old (O'Brien, 1983), most traditional techniques have been until quite recently rather non-specific or, at best, equivocal. Iodine/potassium iodide or periodate-based reactions (such as the periodate/Schiff's procedures) (Jensen, 1962) are exceptions of course, but many others have allowed only empirical assessment of carbohydrate identity or concentrations. With the development of epi-illuminating systems for fluorescence microscopes, and the increased availability of improved bright-field and fluorescent probes for specific functional groups and reactions (Haugland, 1992), it is now possible to carry out relatively specific chemical determinations on microscopic structures.

For the most part we employ the fluorescence microscope for many routine evaluations of carbohydrates in raw and processed food materials. Methods for preparing specimens for fluorometric analysis are quite simple and rarely require anything more than production of hand-cut (razor) or frozen (cryostat) sections ranging from 5–20 μm in thickness. These procedures ensure that compounds of interest are essentially unmodified and unextracted, suffering only minor damage during freezing. Complete details of suitable methods for fluorescence analysis of carbohydrates, as well as proteins, lipids, enzymes, phenolic compounds and other substances are described elsewhere (Fulcher *et al.*, 1989). For high resolution examination, samples also may be prepared using fixation and embedding procedures typical of those used in traditional electron microscopy; these may involve aldehyde fixation and methacrylate or epoxy embedding to allow production of thin (0.1–2.0 μm) sections.

Detection procedures

Fluorescence methods

Complete details of many fluorescence procedures for carbohydrate detection are listed elsewhere (Fulcher *et al.*, 1989; de Francisco & Munck, 1989; de Francisco, 1989). Additional methods for detection of microorganisms (Sigsgaard, 1989) and mycotoxins (Wood & Mann, 1989) are typical of many other approaches using fluorescence. We have also relied extensively on the application of β-glucan-specific fluorochromes such as Calcofluor for detection of so-

called mixed linkage (1-3)(1-4)-β-D-glucans in oats and barley grains (Fulcher, 1986; Miller, 1992). Figure 1 shows a high magnification view of the intermediate layer which is situated between the germ (top) and starchy endosperm (lower) portions of the mature oat kernel. This layer is composed of compressed cell walls which have been formed during the late stages of grain development; its function is not well understood, but it may act as a capillary system for ensuring that water reaches the germ efficiently during germination. Other fluorescent markers are also available for detection of specific carbohydrates such as (1-3)-β-D-glucans which have been located in barley endosperm tissues using aniline blue as shown in Fig. 2 (Fulcher *et al.*, 1977; MacGregor *et al.*, 1989). Indirect detection of polysaccharides is also commonly achieved by detection of naturally fluorescing compounds such as low molecular weight phenolic acids (e.g. ferulic and coumaric acids) which are esterified to specific structures such as the aleurone and pericarp cell walls in all cereals and other grasses (Fulcher *et al.*, 1972; Pussayanawin, 1986; Alkin *et al.*, 1990). Lectins are also very specific markers for microscopic localization of specific sugar residues and linkages (Miller *et al.*, 1984).

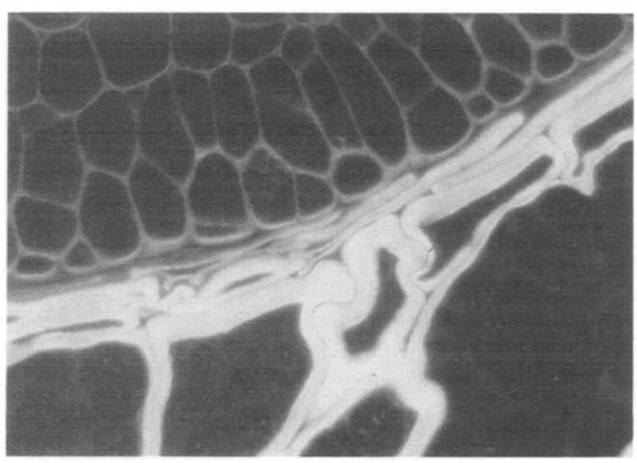

Fig. 1. Fluorescence micrograph of a portion of an oat kernel stained with Calcofluor to reveal β-glucan-rich cell walls in the intermediate layer between the germ (top) and starchy endosperm (bottom).

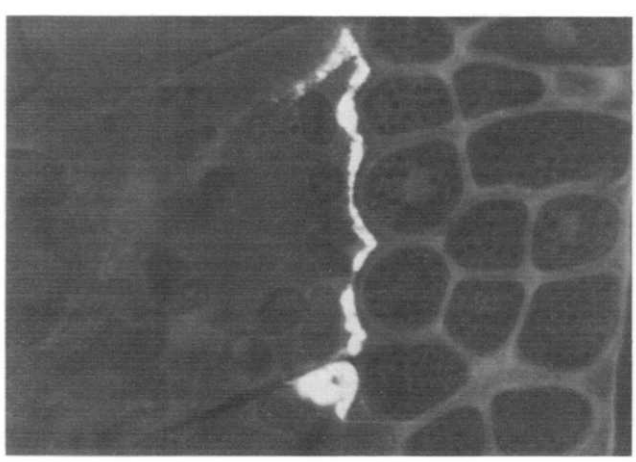

Fig. 2. A fluorescence micrograph of a section of barley kernel after staining with aniline blue to reveal possible (1-3)-β-D-glucans.

Bright-field microscopy

Although fluorescence techniques provide unprecedented sensitivity and very high chemical specificity, bright-field microscopy should not be ignored as a useful technique for visualizing carbohydrate structure and composition. Certainly the periodate/Schiff's method is a reliable indicator of vicinal hydroxyl groups in storage polysaccharides such as starch, and iodine binding to starch granules (Fig. 3) is a historically significant procedure as well. Recently, we have also adapted a relatively unknown dye, Hessian Bordeaux, for use in visualizing starch granules in wheat flour which have been damaged by milling. The dye replaces the more traditional stain, Congo Red, in having a very high coefficient of extinction, and consequently improved detectability (Armstrong, 1992). It also allows routine measurement of the degree of starch damage in diverse flours using image analysis (Dipix Technologies Inc., Canada, personal communication). An example of starch containing both damaged and undamaged granules is shown in Fig. 4 after staining.

Near infrared (NIR) microscopy

FTIR microscopy has been available for some time for analysis of polymers, packaging materials, and for other applications in industry, and its use in analyzing starch and other biological constituents has been explored recently (Wetzel & Fulcher, 1990). However, near infrared techniques are used only rarely in characterizing carbohydrate polymers, although instrumentation for obtaining NIR spectra has been available for several years (Carl Zeiss Ltd, NY). NIR is particularly suitable for OH detection in water and carbohydrates at peak

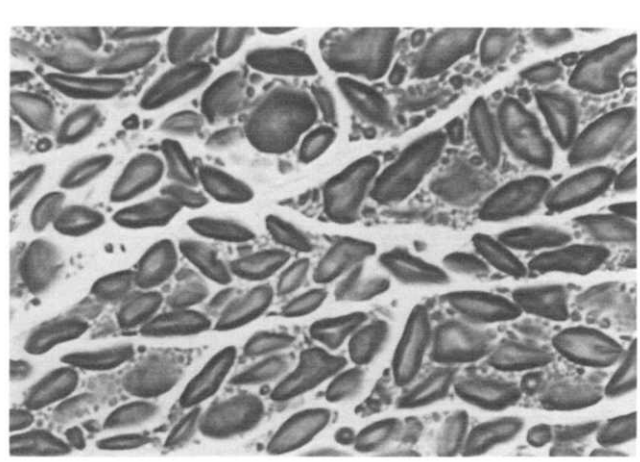

Fig. 3. Iodine/potassium iodide stained section of a wheat kernel showing intense staining in the two starch granule types (A-large; B-small).

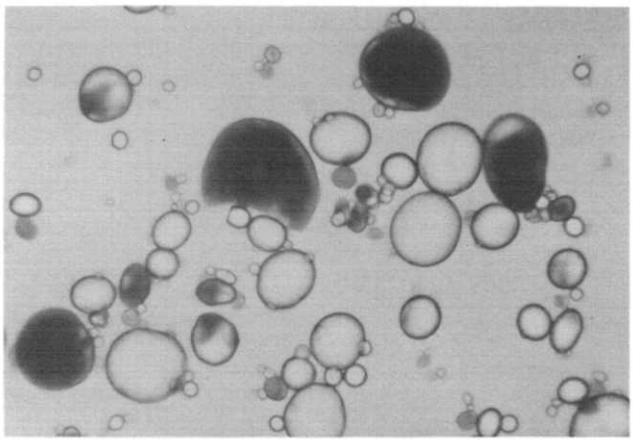

Fig. 4. Wheat starch granules after Hessian Bordeaux staining; damaged granules are intensely stained.

absorbances of approximately 1460 and 1950 nm. Because NIR microscopy offers significant improvements in resolution over magnetic resonance imaging techniques, there may be considerable opportunity for application of this simple technique in characterizing and mapping distributions of water and carbohydrates in raw and processed food materials.

Quantitation

Scanning microspectrophotometry

A scanning microspectrophotometer is essentially a research grade light microscope equipped with an illuminator (halogen, xenon, and/or mercury), a suitable detector (photomultiplier for UV and visible light, or PbS detector for NIR), one or more monochromating systems (preferably grating monochrometers), a scanning stage for moving a specimen through the illumination path, and a computer for control and data handling. For research needs, an ideal system should also be modular, allowing alternating use of different light and detecting systems, and various software formats for spectral, specimen, and kinetic analysis. The UMSP system (Carl Zeiss Ltd, NY) provides such a modular approach in various formats, depending on the number of illuminators, monochrometers, and spectral range required, and we use the UMSP80 routinely in a number of diverse applications in carbohydrate analysis. Details of the operation and examples of applications are provided elsewhere (Fulcher *et al.*, 1989). Uses to date have included measurement of esterase activity in individual cereal cells, *in situ* mapping of phenolic acids, measurement of damaged starch, measurement of quaternary ammonium substitution of individual starch granules (Quinn, 1991), and mapping of protein distribution in individual starch granules (Armstrong, 1992). Further selected examples are included in the following paragraphs.

Spectral scanning—NIR

Figures 5 and 6 show representative spectra obtained by NIR scanning of sections of wheat kernels (approximately 20–30 μm thick) in various states of hydration. Scans were obtained using a NIR monochromator on a

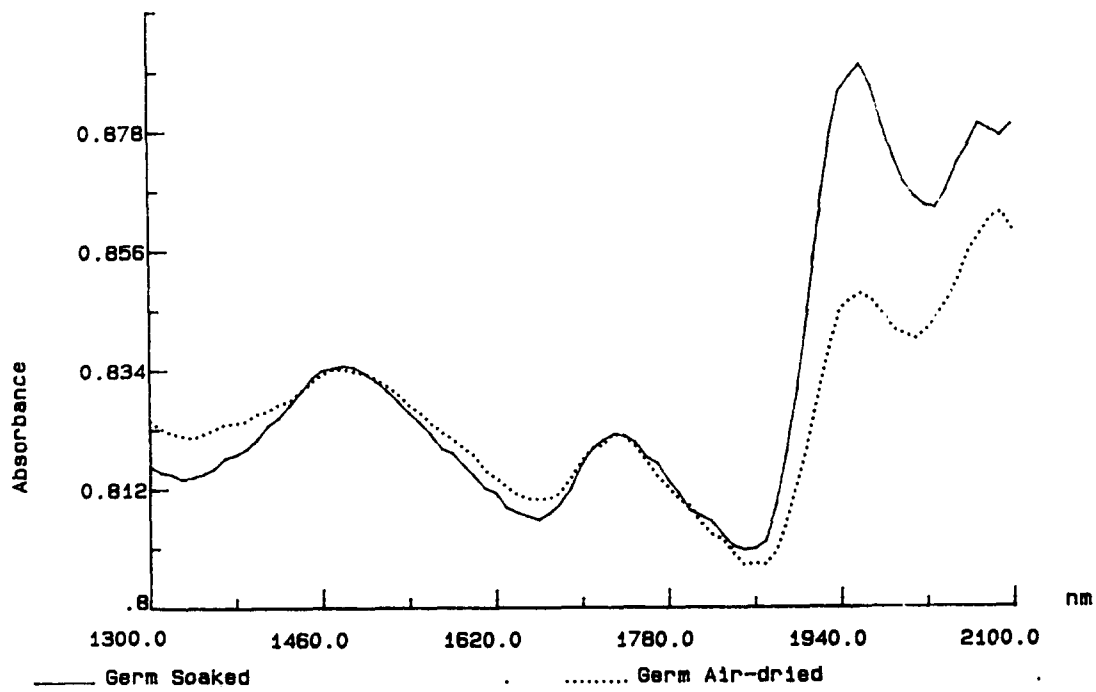

Fig. 5. Means of 20 NIR absorbance spectra taken from the germ region of three different sections. Solid line shows the spectrum after 1 h of soaking and 3 h equillibrium; dotted line shows the spectrum which results from air-drying overnight. Note the large difference between the two spectra at 1950 nm.

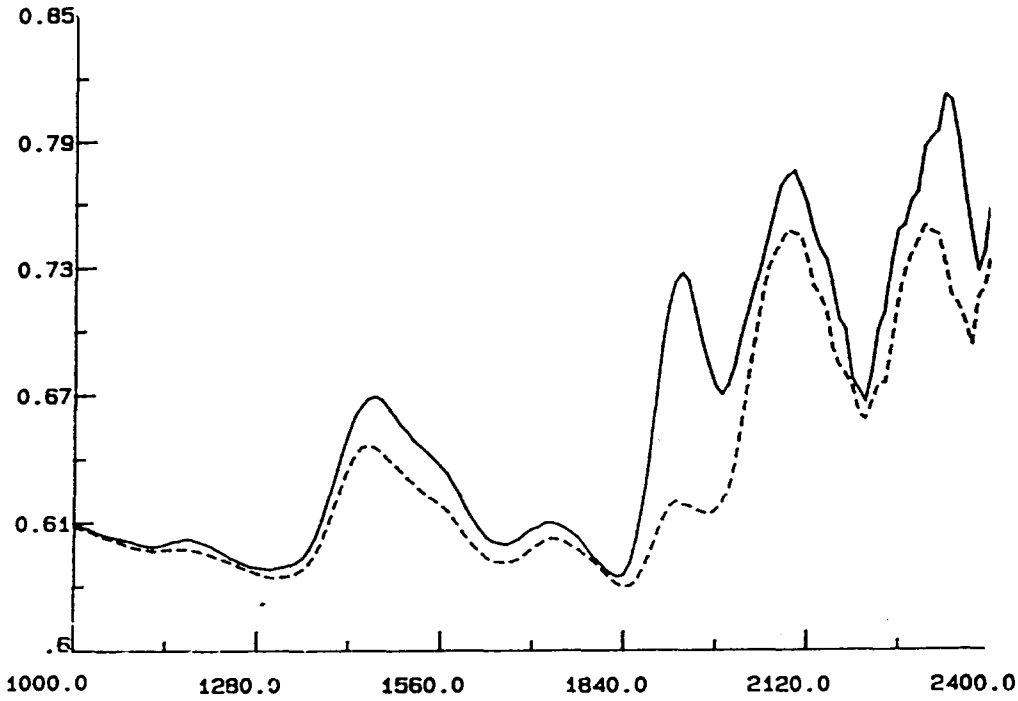

Fig. 6. Similar to Fig. 5, but showing differences in NIR absorption spectra in wheat endosperm cells before and after drying. Again, major differences appear in the spectral region near 1950 nm.

UMSP80, with slits set at 100 nm, and scanning conducted (with 500 ms delay) at 40 nm steps from 1300–2100 nm. Detection was via a PbS detector.

In Fig. 5, spectra were obtained on a section obtained from an intact kernel which was hydrated for 12 h and then air-dried overnight. Absorbance scans were obtained on a portion of the section which would represent an area approximately equivalent to that of a single germ cell. When normalized, the differences between the hydrated and dried specimens are particularly apparent at 1950 nm when the two spectra were normalized at 1450 nm. The 1950 peak represents one of the two major water peaks, and the difference in the two samples is quite striking. Similar differences in single endosperm cells can also be detected in hydrated and dried kernels, and this observation provides an opportunity to evaluate distributions of water in kernels of different types. This is particularly important in view of the ongoing interest and significance of kernel tempering or hydration in the milling process, and further analyses using these techniques should provide insights into the mechanisms and rates which influence water entry into different varieties of wheat during processing. Recently, we have also used this microanalytical approach to characterize oils and carbohydrate-rich syrups in a number of different food applications in which the size of available sample was insufficient for more traditional analyses (e.g. droplets on coated food preparations).

Although NIR spectral scanning is a relatively simple technique, it is rarely used in food analysis. We consider it a useful adjunct to the wide range of bright-field and fluorescence measurements described elsewhere (Fulcher *et al.*, 1989; Miller, 1992).

Distribution of β-glucan in oats

Specimen mapping

Microscopic evidence has indicated that there are differences in distribution of β-glucan in kernels from different cultivars of oats (Fulcher, 1986). These differences, however, have not been well characterized to date. The fluorescent dye Calcofluor, which has been used to detect extracted β-glucan in flow injection assays (Miller, 1992), can also be used as a probe for β-glucan *in situ* to map its distribution in seeds by microspectrofluorometry. In this procedure, oat groats are embedded vertically in blocks of polyester resin, and the surface of the block is abraded to expose kernel cross sections at the desired depth in the seed. After treatment with fluorochrome, the relative flourescence intensity (RFI) of Calcoflour bound to β-glucans is measured by scanning microspectrofluorometry, and is represented as ISO-plots. The ISO-plots are oriented such that the crease is positioned at the left of the section. The RFI of bound Calcofluor is approximately proportional to the amount of β-glucan present (Wood & Fulcher, 1978; Jensen & Aastrup, 1981; Wood & Weisz, 1984; Wood, 1985; Jorgensen, 1988). Figure 7 shows three ISO-plots representing proximal, central and distal scans of a single kernel of

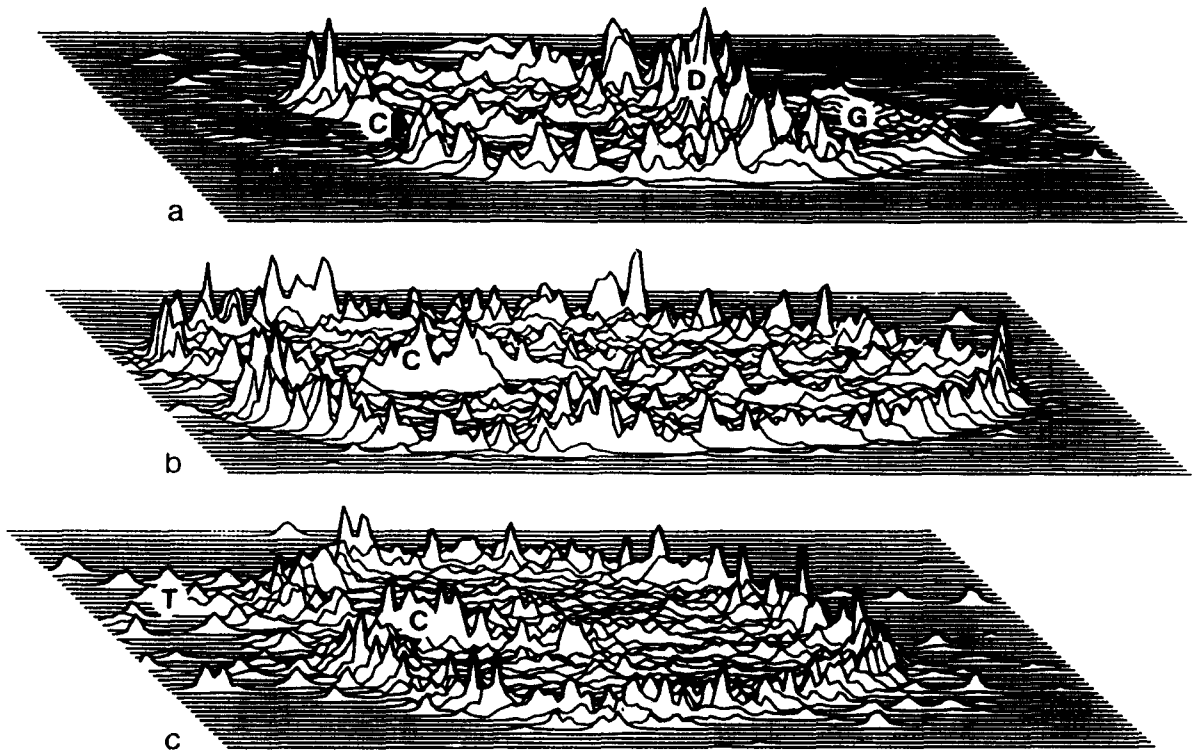

Fig. 7. Distribution of β-glucan in low β-glucan oats, detected by scanning the relative flouorescence intensity of bound Calcofluor to produce intensity (ISO) maps of polymer distribution: a, proximal, b, median, and c, distal kernel ends. G, germ; D, depleted layer; C, crease.

a low β-glucan oat variety taken from a sample assayed at 4.0% β-glucan. In the proximal region of the kernel, which contains the embryo (Fig. 7(a)), the greatest deposition of β-glucan is in the starchy endosperm immediately adjacent to the embryo (sub-embryo area). This area is also called the intermediate or depleted layer, and consists of cell walls left behind as the growing embryo encroached on the endosperm during development of the seed (Brown & Morris, 1890; Fulcher, 1986). The cell walls in the embryo itself are very thin, and contain little β-glucan, as indicated both by the ISO plot and visualized by fluorescence microscopy. A relatively high concentration of β-glucan is also found in the peripheral regions of the kernel, in the layer of cells just inside the aleurone layer. As in the depleted layer, the cell walls in the sub-aleurone layer are also very thick, and wall thickness decreases towards the interior of the starchy endosperm. Microscopic evidence of thinner walls in the interior of the starchy endosperm is reflected in the lower RFI observed in those areas (Fig. 7(a)). Differences in cell wall thickness corresponding to differing β-glucan contents have been reported in barleys with different β-glucan contents (Aastrup, 1983).

Moving from the proximal end into the central region of the kernel, the very large sub-embryo deposition disappears, and the β-glucan distribution is similar to that seen in the ventral portion of the section through the proximal region. The β-glucan content is highest in the sub-aleurone layer (around the periphery of the kernel and up into the crease), with lower levels in the interior of the section (Fig. 7(b)). The distribution in the distal portion of the kernel is the same as in the central region. Small points of fluorescence are also visible outside the kernel at the distal end. These are caused by autofluorescence of the trichomes attached to the kernel. This autofluorescence typically measures less than 5% RFI, which is the lower threshold set for measurements on the microspectrofluorometer, and thus does not interfere with the fluorescence detection of β-glucan. Phenolics in the aleurone layer (which is found around the periphery of the kernel) also autofluoresce at the same approximate excitation wavelength (365 nm) as wall-bound Calcofluor (Fulcher, 1986; Fulcher *et al.*, 1972), as demonstrated in the scan of an unstained oat kernel in Fig. 8. However, the autofluorescence observed in the aleurone cell walls is also of much lower intensity than that of wall-bound Calcofluor, and is below 5% RFI for these experiments. In addition, the autofluorescence in the aleurone layer is quenched to some extent by the use of a non-fluorescent counter-stain.

The pattern of polymer distribution is altered in a high β-glucan (6.4%) cultivar (Fig. 9). The greatest difference is observed in the central region of the kernel where the RFI is high throughout the section and no

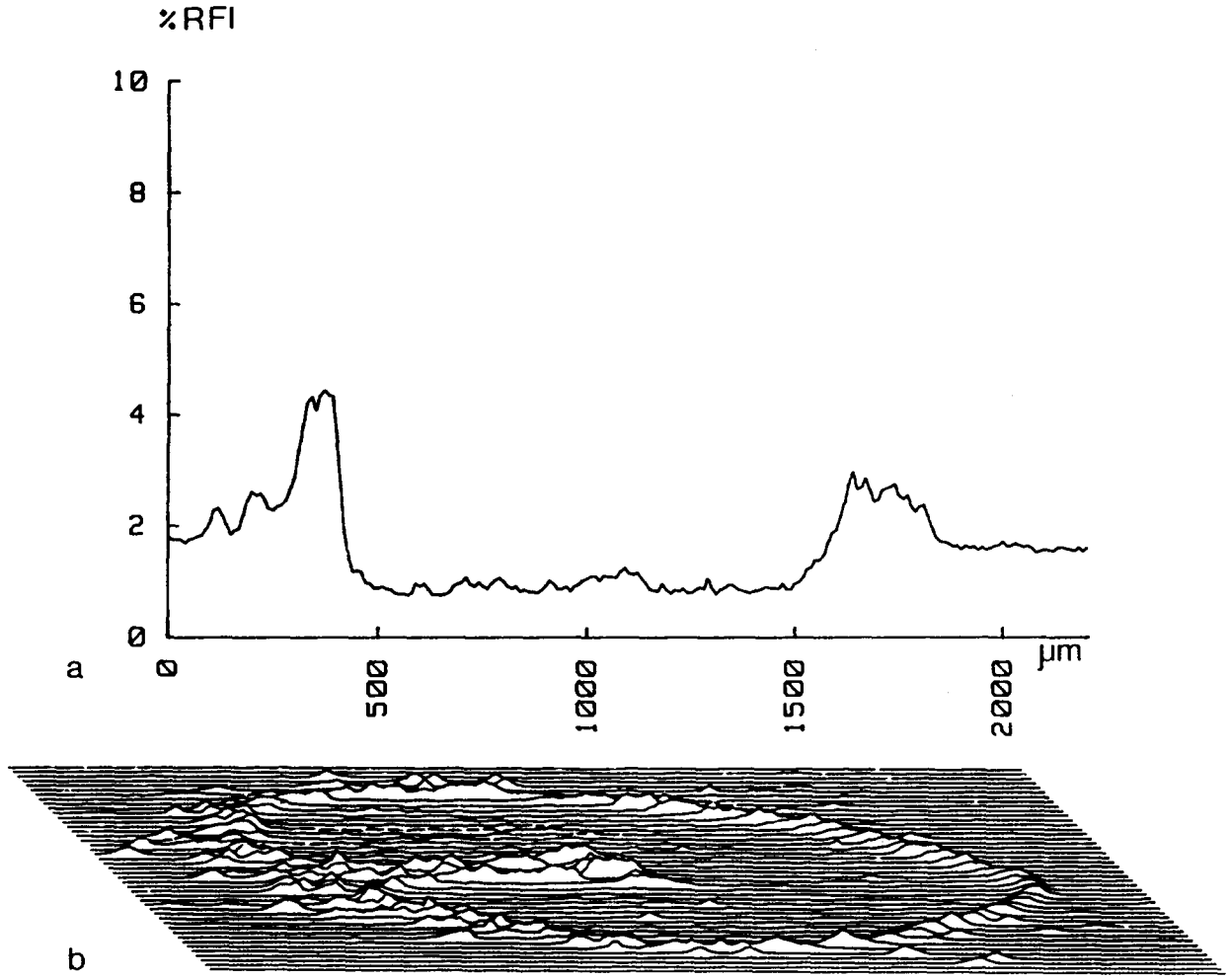

Fig. 8. Distribution of autofluorescence in an unstained (no Calcofluor) section, including (a) mean of 5 scans across the mid-region of the section; and (b) ISO plot of fluorescence distribution.

clear sub-aleurone concentration of β-glucan is observed. The concentration of β-glucan is slightly lower towards the dorsal side of the kernel and a relatively uniform distribution throughout is also observed in other portions of the kernel.

In preliminary experiments with single sections selected from different oat kernels, we have observed a high correlation between the Calcofluor-binding affinities of the single sections (as measured by UMSP80) and flow injection analyses of parent samples. This raises the prospect that single seed determination might be useful in expediting genetic selections for plant breeders interested in raising β-glucan levels in the oat crop. While the equipment required is too complex and expensive to be considered for routine purposes, the possibility of developing single-wavelength devices for this purpose is attractive. The large difference in whole grain β-glucan content observed between high and low β-glucan cultivars has not been reflected in chemical differences in isolated endosperm cell walls (Miller, 1992). Rather, microscopic evidence suggests that the differences in whole-grain β-glucan contents could be attributed simply to differences in concentrations of β-glucan in different regions of the grain, and to size and shape differences in the cells themselves.

Although the distribution of β-glucan varies among the oat varieties we have studied, a consistent feature in each of the cultivars examined is the large concentration of β-glucan in that portion of the starchy endosperm which is immediately adjacent to the embryo. Fluorescence micrographs of Calcofluor-stained sections of this area are similar for both high and low β-glucan cultivars and this consistent concentration of hydrocolloid presumably reflects a requirement on the part of the embryo to maintain a constant moisture level during germination. In some cultivars, particularly those with low β-glucan contents, microspectrofluorometric scans indicated that there was a consistently high level of β-glucan in thick cell walls adjacent to the aleurone layer around the periphery of the kernel.

Thickened cell walls in the sub-aleurone layers of oats also have particular relevance in the production of

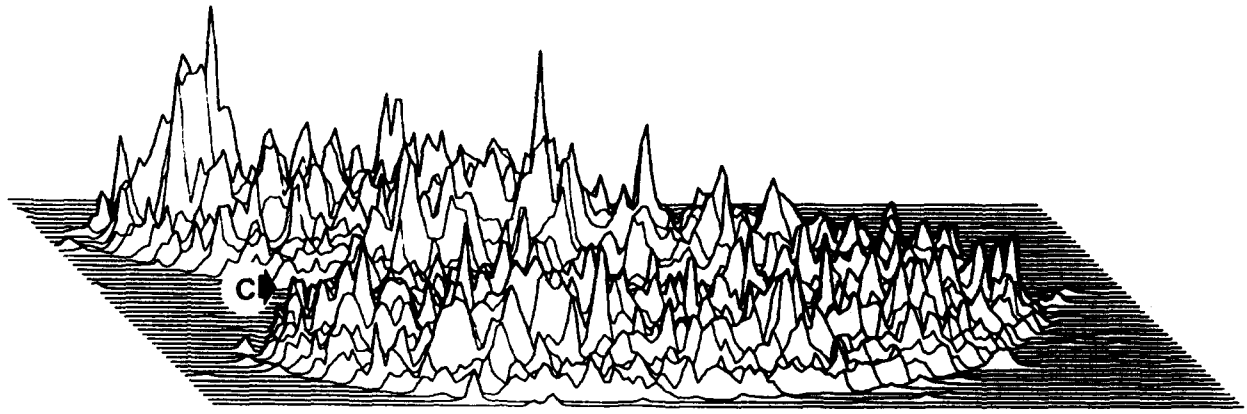

Fig. 9. ISO plot of Calcofluor-induced fluorescence showing β-glucan distribution in section taken from a high (6.4%) β-glucan kernel of oats. C, crease.

oat bran, which is in increased demand due to observed medical benefits which have been reported to accompany inclusion of oat soluble dietary fibre in the diet. Wood *et al.* (1991) reported an average enrichment factor of 1.4 in β-glucan content between whole groats and brans from a selection of oats. Significant changes in ranking, however, were observed in the β-glucan contents of kernels and brans. The differences in distribution observed by Miller (1992) could provide some explanation for these observations. In addition, it has become obvious that scanning microspectrofluorometry presents a valuable tool for selection of appropriate cultivars of oats for production of high β-glucan brans.

Digital image analysis

While it is apparent that spectral and concentration characteristics of cellular materials, including carbohydrates and polysaccharides, can be detected relatively easily and precisely using microscope photometry, it is often equally important to determine the sizes and shapes of important polymeric systems in foods. For this reason, many microscopic methods can be used in combination with digital image analysis (DIA) to allow very rapid and precise measurement of the physical properties of starches, cell walls, and other carbohydrates.

Digital image analysis and its applications in food and grain research have been described recently (Symons & Fulcher, 1988*a*, *b*; de Francisco & Munck, 1989). In particular, Pedersen (1987) demonstrated its application in the measurement of sizes and shapes of barley starch granules, and their relationship to various genetic traits such as differing amylose and amylopectin ratios. Armstrong (1992) has used a similar approach to characterize differences in the ratios of large A and small B granules in wheat starch populations (see Fig. 3), and we have begun to evaluate similar differences in relation to the mixing quality of wheat flour.

DIA employs a simple process of digitization of images obtained from a variety of sources, using an array processor and suitable computer manipulation of the acquired images. The technology is extremely common, it is relatively inexpensive, and it allows rapid measurement of a wide range of microscopic parameters, based on selective detection using specific fluorescent markers. In Fig. 10, the size (area) distributions of A and B starch granules in a variety of hard red spring wheat are clearly distinct; A granules are relatively few in frequency (right portion of the graph) while the smaller B granules are the most abundant. These granules have been visualized microscopically after staining with iodine/potassium iodide and in Fig. 10(a), the solid bars represent the distribution profile in ground whole grain, while the cross-hatched profile represents the distribution in flour after milling. There are few if any differences between the two populations. In Fig. 10(b), however, which represents equivalent material obtained from a different variety of hard red spring wheat, the whole grain starch population (solid bars) is on average very much smaller than that of the milled flour. This is an excellent illustration of the effect of milling on partitioning of granules; as expected, smaller granules are more abundant in the outer regions of some wheat kernels (a product of developmental processes), and milling alters the ratio of the two granule types quite dramatically in some cases. It is to be expected that this simple effect on sizing of the starch population might have detectable effects on dough properties.

DIA is also of some use in developing overviews of structure either at the cellular (microscopic) level, or at the macroscopic level. It is particularly useful in evaluating and mapping distributions of carbohydrates in extruded products, and we have recently begun to use the 3-dimensional reconstruction capability of common software to map distributions of phenolic acid-containing carbohydrates in whole grains. Figure 11, for example is a 3-D reconstruction of serial sections of

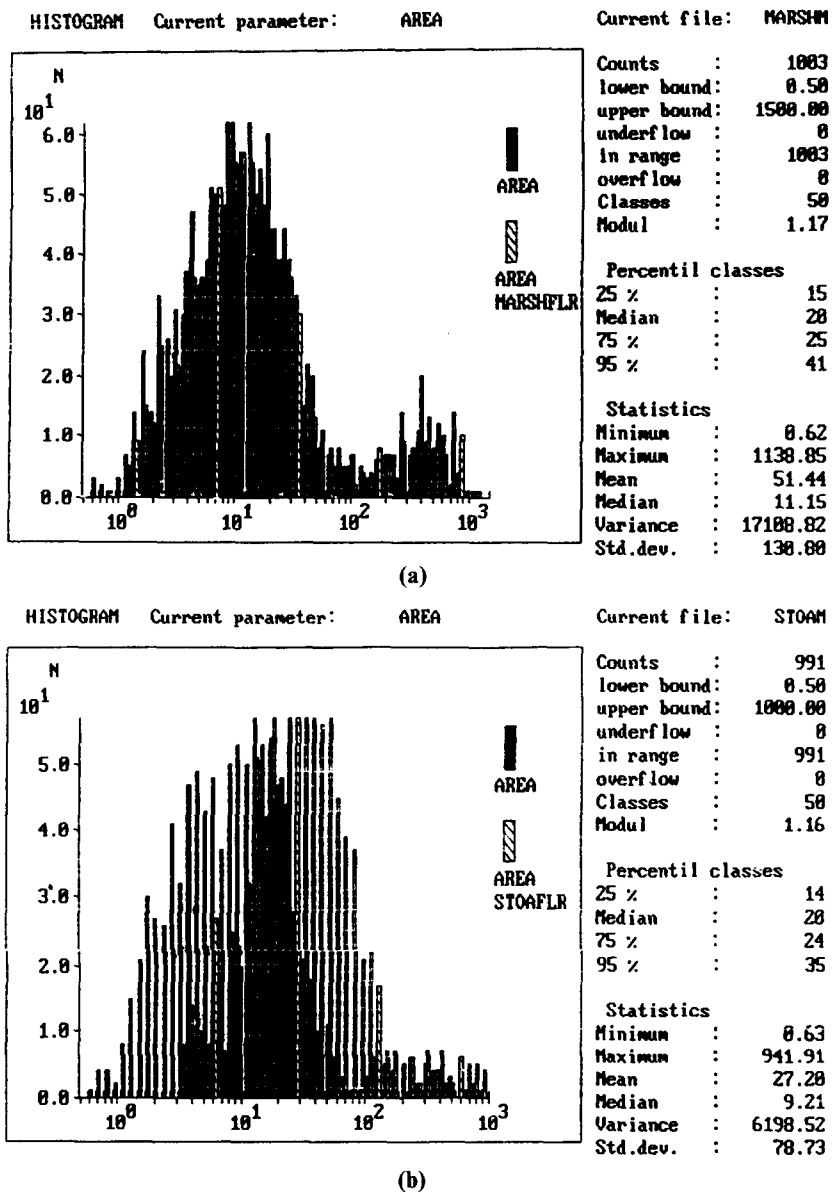

Fig. 10. Frequency distribution of wheat starch granule sizes in flour (solid bars) and grain (cross-hatched bars); each figure (a, top; b, bottom) represents a different wheat variety and in the lower figure, the milling process has changed the relative frequencies of A and B granules dramatically.

a barley kernel. Each section was scanned sequentially at 365 and 450 nm (to excite fluorescence in aleurone and pericarp cell walls specifically) using the UMSP80, and the sections were then reassembled using commercial software installed in a Kontron IBAS image analysis unit. This application is now in use in a number of instances in which patterns of component distribution are reflective of important functional properties.

Using these techniques alone or in combination, protein distribution can be mapped readily in starch granules, modification of cell walls during processing and digestion can be quantified, and cell wall thicknesses and other physical characteristics can also be measured easily. In the latter case, we have begun an analysis of cell wall structure in the oat kernel, and common software for mapping distributions of cell wall-specific probes such as Calcofluor allows ready analysis of cell size and shape, cell wall thickness in relation to quality parameters in oat based foods, and especially analysis of genetic variation in diverse oat types. A typical 'map' of individual cells in the oat endosperm is shown in Fig. 12.

The examples of quantitative microscopy illustrated in this manuscript are but a few of the applications which allow rapid and precise structural and, to varying degrees, chemical analyses of carbohydrates in plants and foods. Many others have been described more fully

in the literature, and new methods are under constant development.

We thank Kerstin Haberer for the use of the starch DIA profiles and Erin Armstrong for providing the photomicrograph illustrating damaged starch.

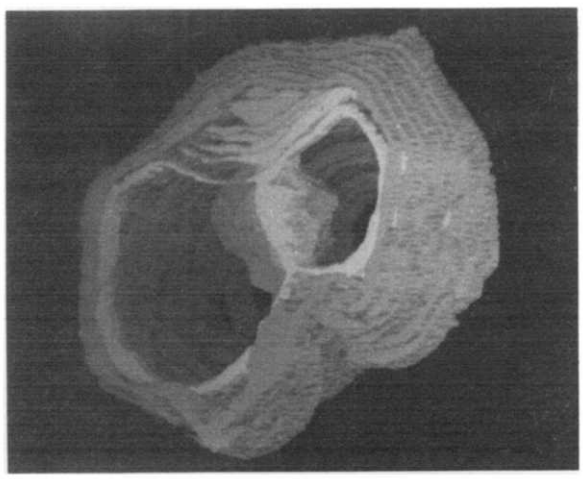

Fig. 11. Partial 3-dimensional reconstruction of a barley kernel after UMSP80 scanning at wavelengths used to excite phenolic acid fluorescence.

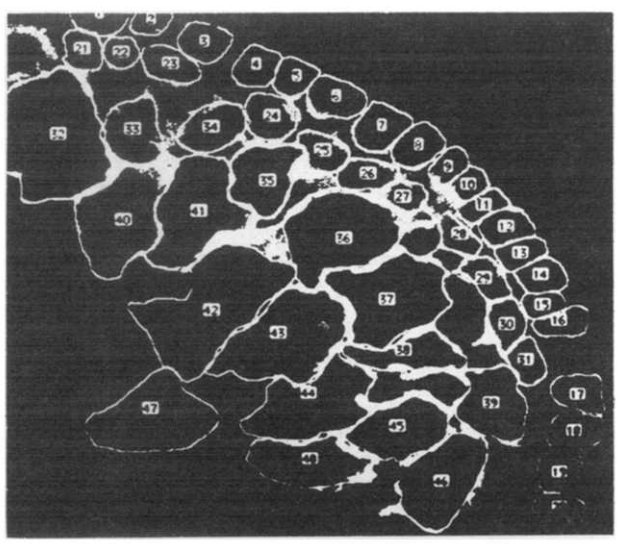

Fig. 12. β-Glucan-rich cell walls enhanced using DIA image extraction software. Cell sizes are readily measured after these enhancements, as are cell wall thicknesses.

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