Strategies for biochemical characterization of cotton fibers

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ABSTRACT

Although the chemical composition of cotton fibers is 95% or more cellulose, cotton fibers are not simple bundles of linear ß-1,4 glucose polymers. Cotton fibers have rather complex structures. Developing fibers contain mono- and disaccharides, soluble and insoluble oligosaccharides, cellulose, and small quantities of pectins, proteins and waxes. During fiber synthesis most of the elongation occurs during the period of primary wall synthesis, however, there is some overlapping with early secondary wall synthesis. The transition from primary to secondary wall synthesis occurs at about 21 days post anthesis (DPA). We have found fibers sampled around 21 DPA particularly useful for investigation of the structure of the secondary wall. The secondary cell wall constitutes the bulk of the cotton fiber and is likely responsible for most fiber properties. The early phase of secondary wall synthesis is a particularly active period of cell wall metabolism. Because the relative concentrations of several carbohydrate pools are rapidly changing during this period, the time between collection and freezing must be minimized, and the samples must be standardized for the time of day collected and the relative nodal position on the plant in order to make valid comparisons between experimental units. We hypothesize that the sugars, other than glucose that are found in developing fibers, perform essential biochemical functions in the synthesis, structure and properties of cotton fibers, and that cotton lint quality is largely an expression of the characteristics of the assembly of fiber within maturing bolls. We are utilizing biochemical methods to monitor the dynamics of simple and complex carbohydrates in maturing cotton fibers. Understanding the dynamics of carbohydrates and glycoconjugates in developing fibers may provide critical insight into the biosynthesis of cotton fiber. Such an understanding is essential to the success of efforts to use transgenic means to improve fiber quality and also may be applied to assist efforts in traditional cotton breeding.

Introduction

Understanding the biochemical composition, mechanism of assembly, and structure of cotton fiber, at the biochemical level, would constitute an invaluable guide to further efforts for the genetic improvement of cotton. Such information might be used to develop biochemical diagnostics to assist classical breeding efforts, and would be essential to directed attempts to transgenically modify fiber properties. The chemical composition of mature cotton fiber is well known (Bertoniere, *et al*., 1993). Cellulose is the principal component, and generally reported as comprising approximately 95% of the mature fiber; however, it has been reported as high as 99% (Maltby, *et al.,* 1979). Cellulose is a ß-1,4-linked glucan, however the organization of the cellulose polymer differs among sources. A minimum crystal unit cell must contain two side-by-side cellobiosyl units (four glucose units). Both crystalline and non-crystalline cellulose are found in cotton, but cellulose typically occurs in cotton fiber as small crystalline micro-fibrils arranged in multi-layered structures (Levin and Pearce, 1998). Less is known about the composition of developing fiber compared to that of mature fiber, and little is known about the manner of its geometric assembly.

Biochemical analysis of developing fiber is more likely to elucidate the mechanism of assembly of fiber and its components than is the analysis of mature fiber. If it were possible to disassemble developing fibers using mild reagents, critical information might be gained regarding intermediate stages of fiber assembly, possible subunits, and the dynamics of possible precursors. Pillonel *et al.* (1980) demonstrated the incorporation of radioactive label from UDPG, GDPG and sucrose into cotton fiber wall polysaccharides. UDPG was the best precursor for ß-1,4-glucans in primary wall but sucrose was by far the best precursor for secondary walls. GDP-glucose served as a substrate for callose, a ß-1,3-glucan. Comprehensive models for the development of cotton fibers have not been presented. The present model for cotton cellulose assembly accounts for only the roles of sucrose, and UDP-glucose as precursors of cellulose (Delmer and Amor, 1995; Delmer, 1999). Sitosterol-ß-glucosides have been produced by isolated fiber membranes, and a role for sitosterol-ß-glucoside as a primer for cellulose synthesis has been proposed, based on the synthesis of sitosterol-cellodextrins (Peng *et al.,* 2002). Thus, with the exception of the possible role of the sitosterol-ß-glucoside, no candidate intermediates have been suggested between and the glucosyl donor and cellulose, a polymer that typically occurs in cotton fibers at a degree of polymerization (DP) of approximately 20,000 (French and Bertoniere, 1993). Cotton fibers develop in two distinct phases, the primary and secondary cell walls, whose synthesis and assembly overlap in time (Benedict *et al*., 1973; Schubert, 1975). In fact, while being a relatively pure form of cellulose, the cotton fiber has a complex structure, and the details of its assembly are relatively unknown.

Experimental procedure

Age of biological samples

Mature fibers may be the tissue of choice for analyses intended to refine utilization of cotton in tex-

tiles or other products; however, analyses of immature fibers could provide more information on developmental events such as the transition from primary wall to secondary wall synthesis. We have used fibers primarily at the age of 21 days post anthesis (DPA), 33 DPA, 45 DPA and from open bolls (mature fibers) for studies on the dynamics of carbohydrate metabolism in developing cotton fibers. Before 21 DPA, distinct and intact fibers cannot be removed from bolls. After boll opening, fiber typically decreases in moisture content to equilibrium with its environment and metabolism is conventionally considered complete.

Composition analysis of mature fiber

A classical cell wall extraction begins with an extraction in water, 0.5% oxalic acid or 0.5% ammonium oxalate at 100 °C for 30 to 60 minutes to remove pectins, typically followed by an extraction in dilute HCl (0.1 to 0.5 N) to remove uronic acids. Next, an extraction in 10 N NaOH for 30 min at 100 °C would remove hemicelluloses. The remaining insoluble products in the strong base would be presumed to be cellulose (Rogers and Perkins, 1968).

Metabolic analysis of developing fiber – field sample handling

If immature fibers are used for analysis, care must be exercised in the collection and handling of the bolls. Field collection and handling must be considered part of the laboratory analysis. Different results were obtained depending on how rapidly the bolls were frozen after harvesting. A comparison was done of bolls frozen by three different protocols: on ice for 1 hr before freezing in a dry ice/isopropanol bath; freezing directly on dry ice followed by sealing in vacuum bags using a Foodsaver®, and immediately immersing in a dry ice-isopropanol bath. The latter method is preferable for soluble mono-, di-, and oligosaccharide analysis since its rapid rate of temperature decline stops apparent metabolic activity in the shortest time (Murray and Munk, 2000a).

Metabolic analysis of developing fiber – extraction of soluble sugar, and oligomeric fractions

We have used a less chemically severe approach to the extraction of cell wall fractions compared to those typically used to extract cell wall components (Murray *et al.*, 2001). The lyophilized fibers were first extracted with water at 0 °C to remove soluble oligosaccharides and monosaccharides (Murray, 1998). Following the cold-water extraction, the fibers were then extracted with 0.1 N HCl in a boiling water bath as shown in Figure 1 (Murray, 2000; Murray *et al.*, 2001). The mono- and oligosaccharides extracted by the cold water procedure include *m*-inositol, galactinol, arabinose, glucose, fructose, melibiose, sucrose, manninotriose, verbascotetraose, raffinose, stachyose, verbascose and tentatively, ajugose which can be used as indicators of fiber development (Murray, 1998, 2000). The oligosaccharides extracted by the 0.1 N HCl procedure can also be used as indicators of cell wall biosynthesis and fiber development (Murray, 2000, 2003).

High pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed using a Dionex Bio LC system with a CarboPac PA-1 column. The eluent was 150 mM sodium hydroxide, isocratic from 0 to 5 min, then a linear gradient from 0 to 500 mM sodium acetate in 150 mM NaOH with detection using waveform for carbohydrates (Murray *et al.*, 2001). To determine the monosaccharide composition of oligomers, fractions were collected from the HPAEC-PAD eluent and passed through a Dionex ASRS-II anion suppressor to remove salt. Fractions were then lyophilized, taken up in 200 ml of water, made up to 2 N trifluoroacetic acid (TFA) (Manzi and Varki, 1993), and flushed with argon and sealed in screw cap plastic vials with O-rings. The samples were then placed in a heating block at 100 °C for 2–4 h. Following hydrolysis, the samples were taken to dryness in a Speed-Vac® overnight and then taken up in 200 ml of water for HPAEC-PAD on a Dionex CarboPac-PA10 column under isocratic conditions in 15 mM NaOH.

Results

Diagnostic value of soluble sugars

We found that classical cell wall extraction protocols applied to mature cotton fibers remove significant quantities of monosaccharides. Glucose and fructose were the major sugars extracted by water or 0.5% oxalate at 100 °C, while at 50 °C and 0 °C the amount of glucose and fructose extracted was greatly reduced while the amount of arabinose extracted increased significantly as shown in Figure 2. These results show that the products extracted from cotton fibers are highly dependent on the extractant and the conditions. They also suggest the chemical and structural complexity of the fibers. Because of such results, the procedures adapted by this laboratory were to begin each analysis using those reagents and conditions least likely to cause general decomposition or distortion of the parent compound, and to proceed successively with stronger reagents or conditions as required for the specific investigation.

The relative quantities of soluble mono-, di- and oligosaccharides extracted from developing fibers in water at 0 °C are diagnostic markers for fiber development as well as indicators of stress on the plants (Murray, 1998). These sugars include glucose, fructose, sucrose, raffinose, stachyose, verbascose, melibiose, manninotriose, verbascotetraose, *m*-inositol, galactinol as well as a number of others. However, several intrinsic variables affect the content of these carbohydrates. These variables must be strictly controlled in any com-

parative investigation.

The content of the major oligosaccharides, sucrose, raffinose and stachyose vary with the time of day as shown in Figure 3 (Murray and Munk, 2000b). Therefore, the protocols used in this laboratory require collection at a standard time of day, generally within one hour of solar dawn. Failure to utilize such diurnal standardization will result in highly variable soluble sugar contents among different times of collection, and also will affect the relative distribution of oligomeric products (Murray and Munk, 2000b). Comparison of cotton lint harvested at dates in September, October, and November indicated that the levels of reducing sugars in cotton lint generally declined as the crop remained in the field (Shaw and Perkins, 1991). In a study with eight varieties, sampled at several nodal positions, and at five time-periods following anthesis, the levels of glucose and sucrose were demonstrated to decline with the developmental age of the bolls (Hague, 2000). In experiments with bolls harvested at the same time from sequential nodes on the same plant, the levels of sucrose and raffinose in fibers varied with the developmental age of the bolls, but these sugars did not vary in the pericarps of the same bolls as shown in Figure 4 (Murray and Brown, 1997).

Also the nodal positions of bolls on the main stem of the cotton plant are also important. There are developmental differences and differences in carbohydrate content between bolls of the same chronological age, when are harvested from the top, middle or bottom fruiting branches (Hague, 2000). Variability in the rate of maturation becomes more apparent among bolls as the season progresses. Fibers about 21 DPA, particularly from the top fruiting branches, may exhibit a great deal of variability in appearance and sugar content as shown in Figure 5, which shows the variability in 25 mg samples. The most compact sample was sticky to the touch while, the one with well-defined fibers did not feel at all sticky, with the other two samples being intermediate. In addition, differences among varieties can be seen in the relative distribution of sugar contents in developing bolls (Murray, unpublished). However, comparative studies among varieties must account for possible differences in diurnal cycles, and rates of development from time of anthesis (for bolls) and from time of initiation (for nodes).

Diagnostic value of extractable oligomers

The glycan oligomers extracted from developing fibers are also subject to similar effects due to extractants, reagents, or reaction conditions as are the soluble carbohydrates. The glycan oligomers vary with the time of day as shown in Figure 6 (Murray and Munk, 2000b). The glycan oligomers that have been extracted also vary with respect to developmental age of the bolls in sequential bolls harvested at the same time on the same plant (Murray *et al.*, 2001). The distribution of the glycan oligomers can also be used as indicators of stress as well as development (Murray, 2000). That the oligomer series varies with the time of day, time of fruit initiation, and with other characteristics is highly suggestive of an assembly pathway (Murray *et al.*, 2001)

Discussion

The ultimate goal is the application of the data obtained from the extractions and characterizations to the task of cotton improvement. We have found differences in carbohydrate constituents among varieties grown in the same environment. These include differences in the relative amounts of soluble carbohydrates, differences in the character and relative distribution of glycan oligomers, amounts of insoluble material released by enzymatic degradation, and different amounts of crystalline cellulose. Fiber properties depend on the biophysics of the microfibrils. The chemical composition and structure of the microfibrils depends on their biosynthetic mechanism. As such, biosynthesis ultimately determines fiber properties as they are commonly measured by the textile industry for guidance in fiber processing and for fiber purchasing. The kind of diagnostic protocols that we hope to refine would be a means to understand the process of fiber assembly. The objective is to develop a means of biochemical characterization that can be related to fiber quality determinations. Such information could then be applied to crop physiology investigations, classical variety selection, or be used as an invaluable analytical adjunct to assist efforts to transgenically improve cotton output traits. Increased understanding of the pathway of cellulose synthesis in cotton fibers, assembly of the microfibrils, and effects of external factors on fiber development may be essential to interpreting the results of attempts to modify fiber properties by genetic means.

Further research is on-going with specific enzymes to elucidate the structure of cotton fibers at various stages of development (Murray and Nichols, 2002). Enzymatic degradation is a less disruptive and more specific means of partially disassembling developing fiber than is the use of the mild chemical means previously described. Enzymatic degradation may reveal carbohydrate subunits that are close to the native state in which they occur in the developing fibers. As such, they may also be subunits in fiber assembly. We anticipate congruence between carbohydrates that may be extracted from developing fiber and that may be recovered from fiber by enzymatic means.

The working hypothesis is that soluble components are assembled to insoluble particulate precursors that are incorporated into the fiber wall. We hypothesize that a portion of the cellular sucrose pool is converted to raffinose, and subsequently to the other sucrosyl oligosaccharides. These oligomers then may be incorporated into larger molecules. Their fructosyl moieties could be removed by cell wall invertase (Sturm

and Chrispeels, 1990; Sturm *et al.*, 1995). The galactosyl donor for the synthesis of these oligosaccharides is galactinol (Kandler and Hopf, 1982). Galactinol is ubiquitously present in these extractions and is known to function in a similar fashion in other systems (Richter *et al.,* 2001). Precursors for wall synthesis may enter the wall from the extra-fibrillar space as well as from the inside of the fiber membrane, as first suggested by Delmer *et al.* (1974).

Through systematic analysis of developing and mature cotton fibers by the methods described here and other methods yet to be developed, it should be possible to describe fiber development at the biochemical level and to correlate such data with conventional fiber quality measurements. In addition to elucidation of the biosynthetic pathway, it should be possible to identify characters that will be useful indicators for fiber quality improvement. Consequently, this approach should provide useful aids for the genetic manipulation of cotton to produce higher quality fiber.

Acknowledgement

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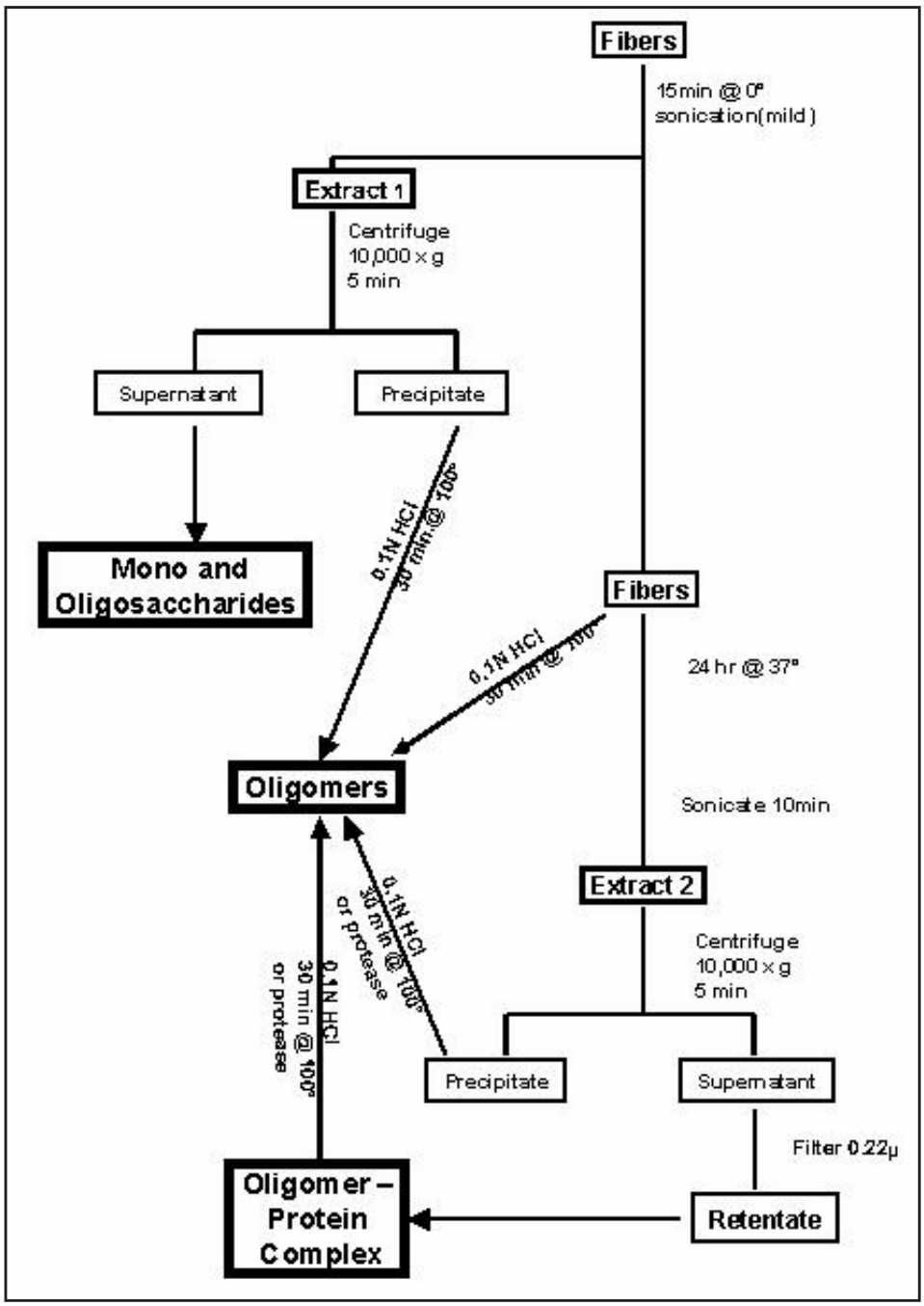

Figure 1. *Extraction procedures for cotton fibers (Murray* et al., *2001).*

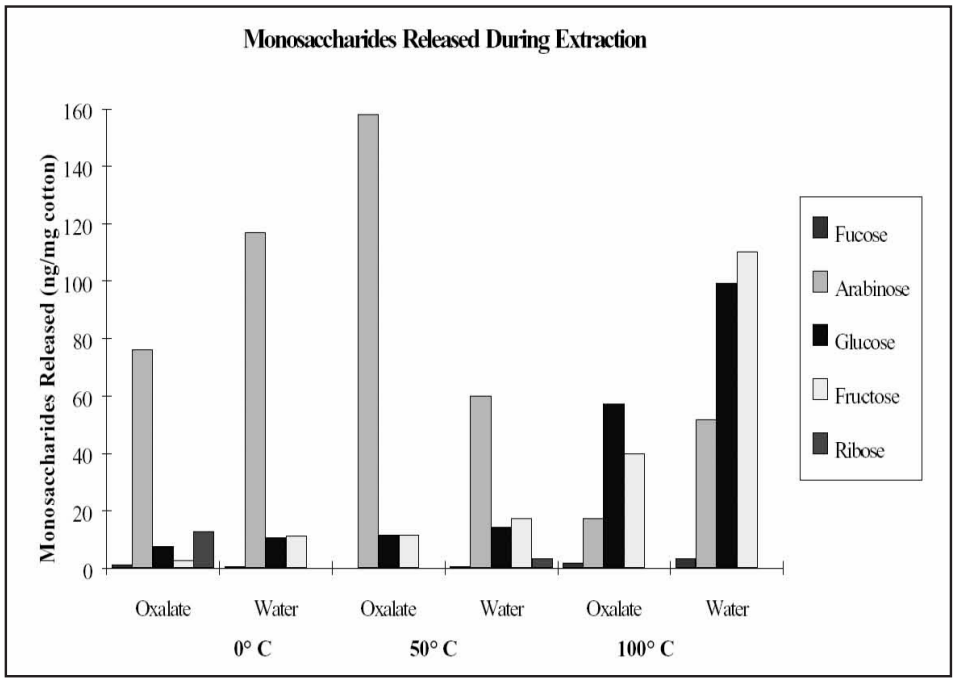

Figure 2. *Monosaccharides extracted from mature cotton fibers under different conditions. In each case from left to right are values for fucose, arabinose, glucose, fructose and ribose.*

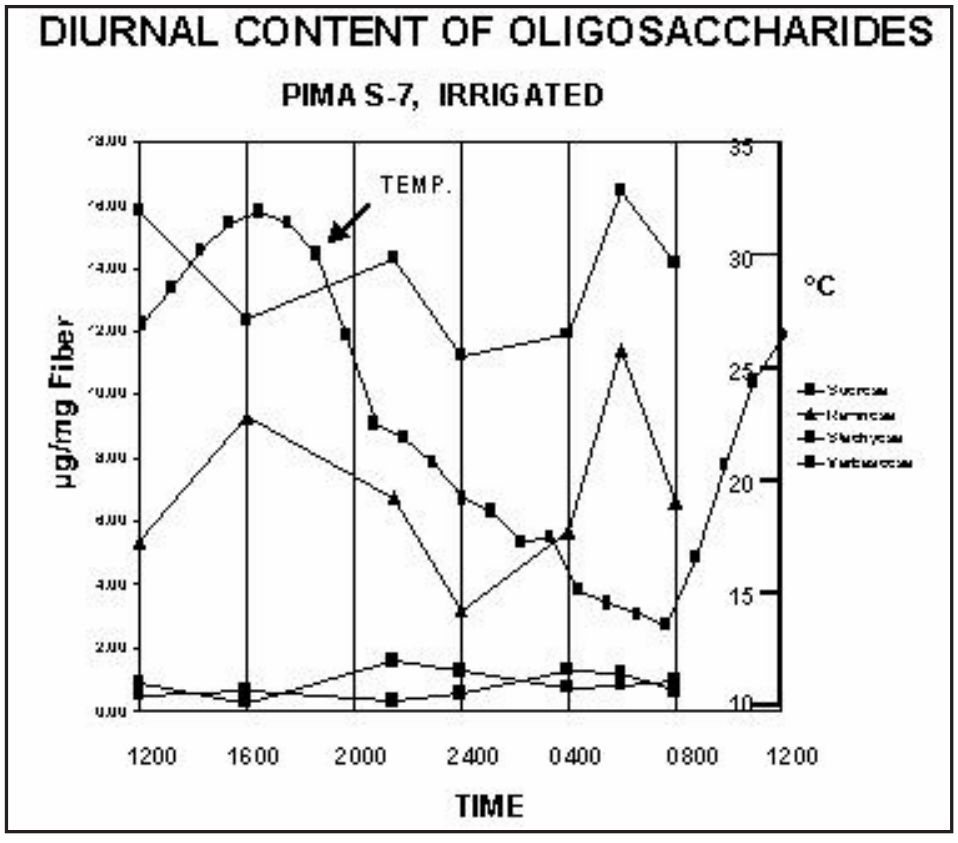

Figure 3. *Diurnal variation in soluble oligosaccharide content of fibers (Murray and Munk, 2000b).*

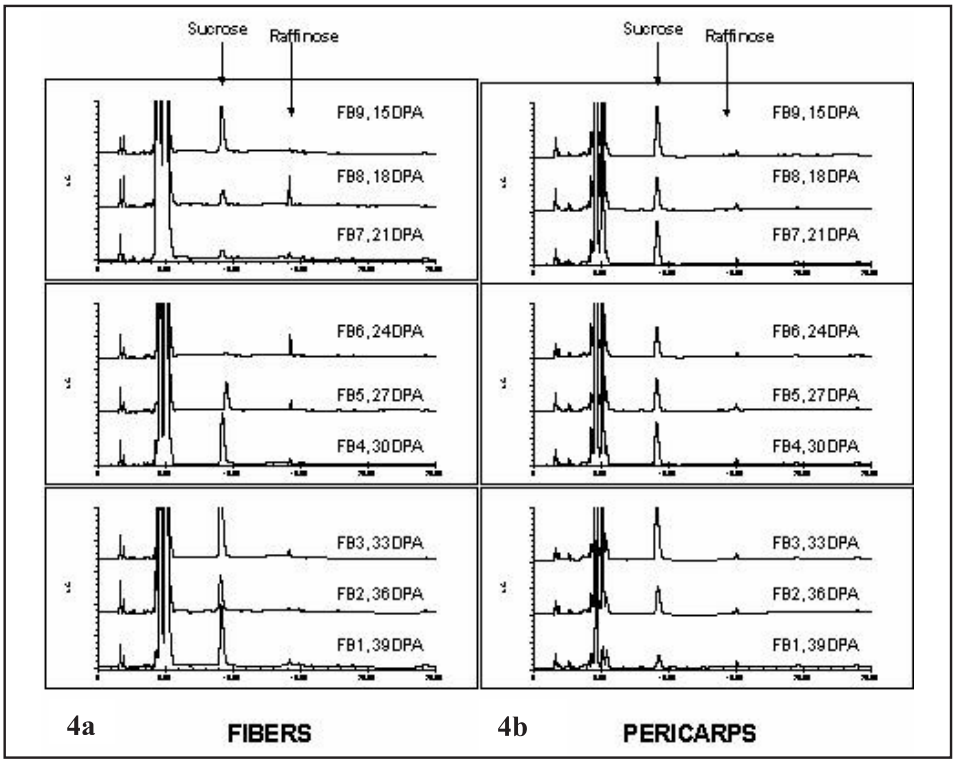

Figure 4. *Levels of sucrose and raffinose in fibers (4A) vary with developmental age of bolls. From 18 to 21DPA, sucrose decreases and raffinose increases. Levels of sucrose in pericarps (4B) of the same bolls. Pericarps were used as a control tissue to reflect sucrose availability at the boll so sucrose variability is due to utilization in the fibers.*

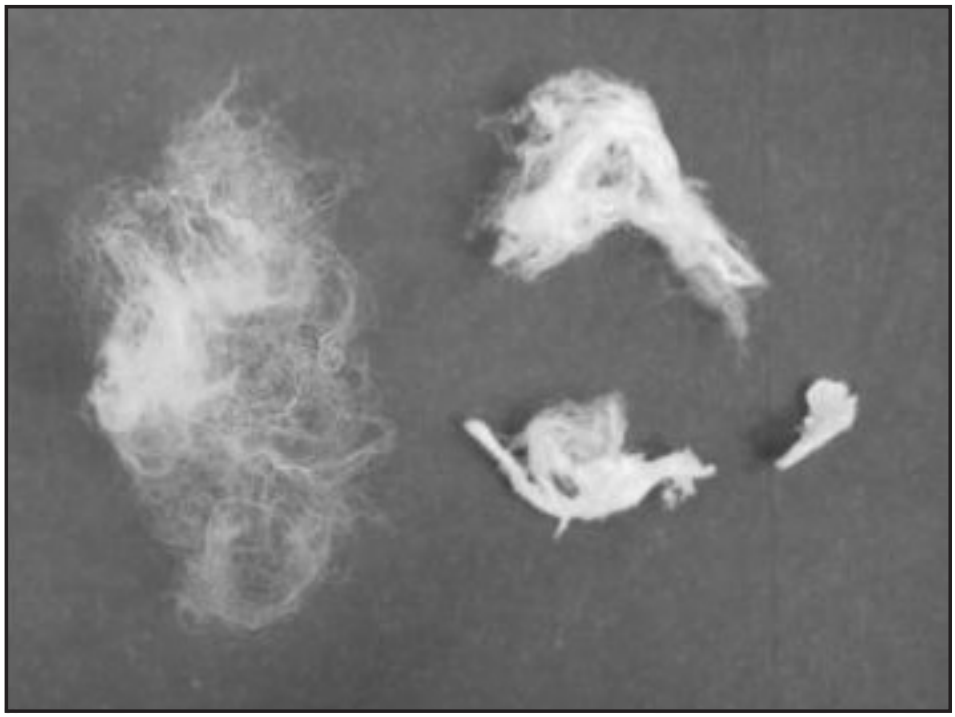

Figure 5. *Example of variability in 21DPA fibers from the top zone of the plant. Each sample is 25 mg.*

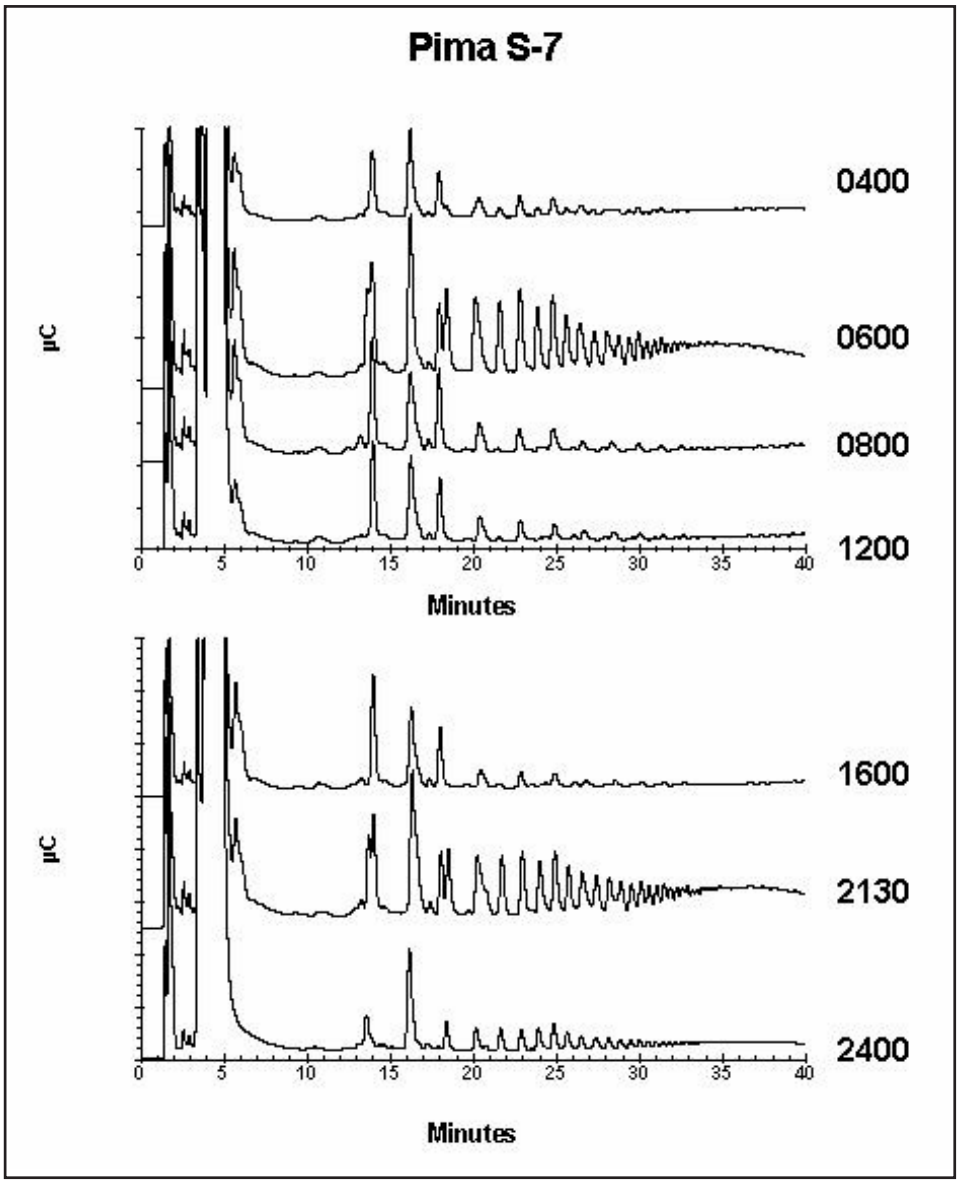

Figure 6. *Variation in content of glycan oligomers extracted from fibers at different times of the day (Murray and Munk, 2000b).*