The Need for Insecticide Applications on Dual-gene Insect-resistant Biotech Cotton in the US Production System

In the United States, *Helicoverpa zea* is a major pest and is commonly known as the bollworm when it affects cotton. However, when *H. zea* attacks other crops, the caterpillar is often named after the host plant, e.g. corn earworm on corn, sorghum headworm on sorghum, soybean podworm on soybean, tomato fruitworm on tomato, and others. The wide range of hosts and the sequence of crops that the insect feeds on over a single growing season have a significant impact on its potential to develop resistance to the toxins expressed in Bt cotton and other biotech crops. This polyphagic quality gives rise to seasonal developmental scenarios where only a limited number of generations would not be exposed to the transgenic toxins, whether in Bollgard or Bollgard II. Furthermore, the use of similar Bt toxins in both Bt corn and Bt cotton may subject populations to multiple selection exposures within a single year. The commercialization of more biotech crops carrying the same Bt genes is going to increase the risk of developing resistance to the toxins. The tobacco budworm *Heliothis virescens* is a major pest and has always required a greater number of insecticide applications than *H. zea*. This situation changed, however, with the introduction of Bollgard cotton in 1996. Bollgard biotech cotton eliminated 100% of the applications for tobacco budworm, but supplemental control of the bollworm remained a routine practice until Bollgard II was adopted. Consequently, the threshold for bollworms was revised to control the escape population. Adoption of Bollgard II enhanced in-plant control of caterpillar pests, particularly the bollworm. Later, in 2005, Dow AgroSciences made available an alternate dual-gene technology, WideStrike®. While varieties with Bollgard II® or WideStrike® technology provided very good control of caterpillar pests, they do not offer 100% control of bollworms.

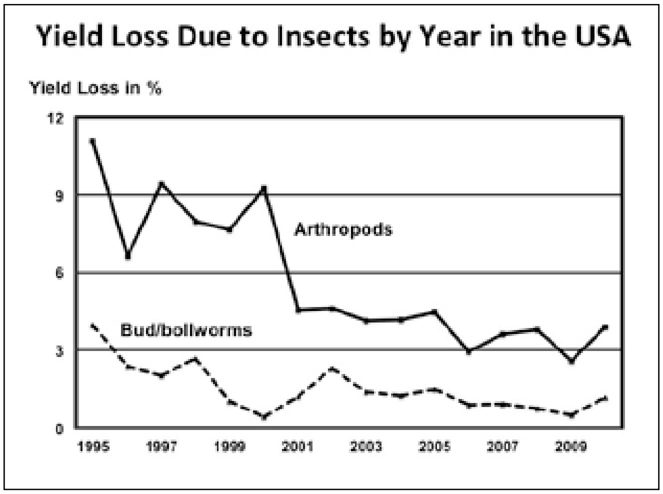
The situation with the fall armyworm *Spodoptera frugiperda* differs significantly from that of the tobacco budworm and the bollworm. The fall armyworm is an occasional-to-sporadic pest in many cotton areas around the world. It is also known to be a migratory pest in the USA. The pest attacks the bottom part of the plant, where it lives, thereby making it difficult to detect infestations using standard sampling protocols. Also, reactive insecticide sprays often yield inconsistent results. There have been questions concerning the performance of transgenic Bt cottons against this pest. WideStrike® provided better protection than either Bollard or Bollgard II cottons against the fall armyworm.

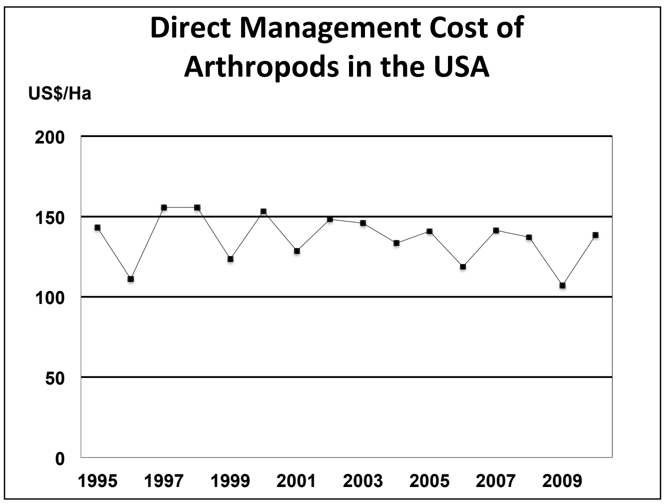
The other two-fruit damaging pests on cotton in the USA, where biotech varieties were adopted sooner than in any other country, are the pink bollworm *Pectinophora gossypiella*, and the cotton bollworm *Helicoverpa armigera*. The cotton bollworm, an American bollworm, is not a serious pest on cotton. The cotton bollworm is typically more difficult to control with Bt cotton than other targeted lepidopteran pests and often requires spraying with insecticides as a supplemental control measure. Protection against the pink bollworm improved with the introduction of dual-gene insect-resistant biotech cotton.

Losses due to Insects in the USA

In the USA, losses due to arthropods have been assessed since 1979. Damage is expressed in terms of loss in yield attributable to various individual pests. The necessary information is provided by county agents, extension specialists, private consultants and research entomologists. All data are averaged over the total area of each reporting unit. For example, if a unit report comprises 100 hectares and there has been an 8% loss on an area of 25 hectares, the average loss would appear in the data as a 2% loss. This averaging procedure is used on all reported data, including yields and control costs. Yield losses due to arthropod pests have diminished significantly in the last 15 years. Since the introduction of biotech cotton, loses due to budworms and bollworms have also declined, from 3.97% in 1995 to 1.2% in 2010 (Anonymous, 1996-2011). Losses due to certain other insects, such as *Lygus* and stink-bugs, have remained almost at the same level for the last 15 years. However, there have been high year-to-year fluctuations, which is also true for bud/bollworms. Yield losses due to bud/bollworms were only 0.5% in 2009/10. Based on the average yield data for 1995/96, it is estimated that 66.7 kilograms of lint per hectare were lost due to arthropods in that season, compared to 36.0 kilograms in the 2010/11 season. Thus, it may be inferred that the most recent pest control measures have not only saved on insecticide costs but have also contributed to lower losses and higher yields.

In the USA, data are also collected on direct management costs in connection with arthropods. The data appearing in the followng charts show that despite increased pressure from certain sucking insects and despite price hikes in insecticide products, the direct management cost of arthropods has not increased in the last 15 years (Anonymous, 1996-2011). In 2010/11, the cost of insect-resistant biotech cotton is estimated at about US$35/ha, which represents about 25% of the cost of arthropod management. There are many factors responsible for the lack of increases in the cost of arthropod control over the past 15 years. Biotech cotton is one of the major factors, but the boll weevil eradication program has also significantly





reduced boll weevil damage, particularly in Texas. Boll weevil infestation, which covered up to 0.9 million hectares in 2002, has been brought down to less than 50,000 ha in 2010.

The Resistance Issue and the Need to Spray

The year 2010 was the fifteenth anniversary of the introduction, adoption and large-scale commercial use of biotech cotton in the evolutionary quest to control insect damage in cotton. One of the most convincing lessons learned from the use of insecticides around the world as applied to biotech cotton was that the entire industry had to design resistance management programs for biotech cotton even before the first single-gene insect-resistant biotech cotton was commercialized in 1996. The dual-gene technology Bollgard II® (Cry1Ac + Cry2Ab) introduced by Monsanto in 2003 completed its first eight years of commercial planting in 2010. Two years later, another dual-gene, insect-resistant biotech cotton, WideStrike® (Cry1Ac + Cry1F), was introduced and commercialized by Dow AgroSciences and it completed its first six years of commercial use in 2010. Monsanto subsequently discontinued commercial production of Bollgard (Cry1Ac gene) cotton for fear that insects might develop resistance to a single–gene variety faster than to a duel–gene variety.

The experience with dual-gene technology in the USA has shown that cotton varieties with currently available multiple Bt gene technologies may provide very good control of caterpillar pests, but they may not offer 100% protection against the bollworm. Many researchers have observed that under extreme natural pressure from bollworms, insect-resistant biotech varieties might display inconsistent control of target bollworms and might require supplemental applications of insecticides to avoid yield losses due to bollworm damage. In 2010, Greene (2011) experienced the highest recorded pressure from *H. zea* in field trials run in South Carolina over the last five seasons. Trial fields of existing and promising biotech cotton technologies were inundated by natural infestations of bollworm, and variable results were observed. Greene (2011) noted peak boll damage levels approaching 20%, 60%, and 30% in unprotected varieties with Bollgard II®, WideStrike®, and TwinLink™ traits, respectively. The single-gene technology Bollgard® showed as high as 60% bollworm damage at the peak of bollworm pressure. He reported that research is underway to develop treatment thresholds designed specifically for multiple Bt gene technologies as they become available.

Jackson *et al.* (2011) observed that during the last few crop years, dual-gene insect-resistant biotech cottons (Bollgard II® and WideStrike®) have increasingly required greater numbers of insecticide sprays targeting the bollworm. Biotech cotton in Mississippi received an average of 1.7 applications per hectare in 2009 for supplemental control of bollworms; the figure was increased to 2.3 sprays per hectare in 2010. Pheromone trap captures of adult bollworms have also shown catch rates almost two times higher in 2010 than in the previous four years. The seasonal average number of bollworm moths captured per trap per week was <50 for 2006-2009 and >100 moths per trap per week in 2010. Researchers collected bollworms from resistant biotech cotton and corn (i.e. Bollgard II®, WideStrike®, and SmartStax®) and tested them for susceptibility to Cry1Ac and Cry2Ab through diet-incorporation, dose-mortality bioassays. A laboratory colony of the bollworm (LabZea) that is susceptible to various Bt toxins was assayed as a control line. Resistance ratios for Cry1Ac indicated that bollworm populations collected from pyramided-gene Bt crops were 3-8X less susceptible to Cry1Ac than the laboratory-susceptible colony. Susceptibility rates of these colonies to Cry1Ac was comparable to many of those found during 2002-2008 in Arkansas, where resistance ratios ranged from about 0.1 to >500. As with the Cry1Ac susceptibility estimates, the Cry2Ab resistance ratios ranged from 2-12.

Mortality ratios generated from the % mortality of a colony subjected to discriminating doses of either 100 μg/ml of diet (Cry1Ac) or 150 μg/ml (Cry2Ab) showed that Cry1Ac-susceptibility remained unchanged from 2002-2008. However, the mortality ratio for Cry2Ab in 2010 was 0.4, which was lower than the range of 0.6-1.0 between 2002 and 2008 (in the state of Arkansas). These data suggest that, whatever reduction in susceptibility is being observed, it is most likely due to reduced susceptibility to the Cry2Ab protein. This, however, would not explain increased survival of bollworms on WideStrike cotton varieties, which produce both the Cry1Ac and the Cry1F Bt proteins. Jackson *et al.* (2011) have also pointed toward another factor that could be responsible for higher survival on dual-gene biotech cotton. They suggest that greater population densities of bollworms during the season could be the cause of the increased number of survivors observed in certain fields planted to biotech varieties.

Siebert *et al.* (2011) of Dow AgroSciences LLC concluded that their WideStrike varieties, as opposed to non-Bt varieties, showed a multi-year mean reduction in boll damage using WideStrike at 82% at each location. They saw a difference in the damage done by bollworms on insect-resistant biotech cotton, but no one claims complete control of all bollworms. The study of reference showed an 82% reduction in bollworm damage, but that only means that there was 18% damage that might be avoided either through more perfect biotoxins or by the application of insecticides. Some other studies found slightly higher mortality rates, i.e. 85.4%, against the bollworm complex, including: beet army worm *Spodoptera exigua*, cabbage looper *Trichoplusia ni*, bollworm and fall armyworm *Spodoptera frugiperda*. The single-gene Bt cotton produced a mortality rate of 45.3%. Siebert *et al.* (2011) also found no trends toward a change in efficacy over a given time span. According to Siebert *et al.* (2011), the contributing factors explaining the levels of boll damage in a WideStrike variety in the absence of supplemental foliar sprays may include: 1) intensity and duration of bollworm infestations; and 2) Cry protein expression patterns during periods of bollworm pressure linked to soil moisture and daytime and nighttime temperatures. The Dow AgroSciences LLC team found no evidence that decreased susceptibility to Cry1Ac or Cry1F over time is a factor leading to greater plant damage. Researchers concede that supplemental sprays targeting bollworm have always been occasionally necessary in WideStrike cotton, particularly against high densities and/or sustained infestations.

Transgenic cotton with Bt genes has reduced the need for conventional insecticides, while providing benefits for human health and the environment. For example, in U.S. cotton, the average number of insecticide applications used against the tobacco budworm and the bollworm complex decreased from 5.6 in 1990-1995 to 0.63 in 2005-2009 (Williams, 2008-2010). It is advised that varieties containing WideStrike should continue to be scouted for the bollworm. When supplemental insecticide treatments are warranted, appropriate insecticides and application rates should be selected and timed appropriately to manage infestations.

Endotoxin Expression

Genes determine all physiological and morphological traits and their ultimate impact on living organisms. A gene is a basic unit that determines variation/diversity and similarities/heredity and is defined as a DNA segment containing a specific sequence of nucleotides. All genes in all living organisms express themselves through proteins or enzymes. The expression of a gene varies according to the sequence of nucleotides, the nature of their promoter, their insertion site in the modified plant, the plant's internal environment and the different sources of modification (biotic and abiotic). Transgenes are able to fully express more perfectly when conditions are optimal. Thus, there are many factors that might determine the need for additional sprays on dual-gene varieties. These factors directly and indirectly influence the amount of endotoxin expressed in each biotech cotton, as well as the insects' reaction in terms of sensitivity and/or tolerance to the toxins produced within the plant. Some of these factors are discussed below:

- Different lepidopteran species vary in their susceptibility to endotoxin proteins. Some larvae will continue to live for two to three days after feeding has stopped. Individual instars may damage different plant parts: squares/buds, small bolls or large bolls. For example, in the case of the fall armyworm, 3rd, 4th, and 5th instar larvae damaged bigger bolls less than they did mid-size or smaller bolls. It has also been shown that damage to squares by all instars resulted in a significant reduction in survival of fruit to harvest. Insect feeding on large bolls did not reduce the probability of survival of fruit to harvest; however, yield from damaged bolls may be much lower compared to yields from unaffected bolls. It is generally accepted that the fall armyworm is one of the lepidopteran species that is least susceptible to the Bt endotoxin proteins expressed in cotton.
- Endotoxin concentrations need to be quantified because the amounts of endotoxin found in the leaves and in the other parts of the cotton plant vary significantly. Thus, the ultimate efficacy of any particular single-gene or multiple-gene biotech cotton will depend on the protein expression levels in different plant parts (Adamczyk *et al*., 2008). When larvae feed on less effective Bt type cotton leaves, they need to consume greater quantities of leaf material to ingest the amount of endotoxin needed to be lethal for them. It has also been established that feeding on transgenic cottons significantly reduced pupal weight and emergence, and also delayed larval development. According to Kranthi *el al.* (2005) toxin levels decrease as the crop matures and is consistently very low or undetectable in squares. *H. armigera* and bollworm larval mortality was greater on leaves containing toxin than on other fruiting parts (Arshad *et al*., 2009). The amount of toxin may also vary among the parts of a single plant. In general, petals, leaves and squares have higher

Expression of Cry1Ac Endotoxin in Various Cotton Varieties at Two Locations

Variety	Concentration of Cry1Ac Endotoxin (ppm)	
	Mississippi State	Stoneville
DP 33B/458B/RR	2.03 a	2.95 a
Sure-Grow 125 BR	1.15 b	2.69 a
PM 1218 BG/RR	0.90 bc	1.87 bc
ST 4892 BR	0.64 bc	1.94 b
DP 451 B/R	0.76 bc	1.49 c
ST 4691 B	0.61 c	1.56 bc

concentrations of Bt toxins than anthers and ovules. Research has also shown that bolls in position 1 (close to the main stem) have higher concentrations of toxins than those in positions farther away from the main stem. Concentrations of Bt proteins steadily decline between nodes 9 and 17. The age of the plant is also important as some data show that toxin amounts decline in older plant parts, particularly at 110 days after planting. This situation will demand insecticide sprays against target bollworms and those that may be prevalent at the crop maturity stage. Chloroplast concentration is also claimed to have an effect on toxin expression and, consequently, on sensitivity to protection against target insects, particularly *Spodoptera frugiperda*.

- Different varieties may express different amounts of toxin. As a consequence, the efficacy of toxins may differ widely from one variety to another (Kranthi *et al*., 2005). Hofs and Vaissayre (2007) have done an extensive review of the factors responsible for toxin expression. The work done in the USA as early as 2001 clearly revealed that there were significant intervarietal and interlocational differences in toxin expression (http://msucares.com/newsletters/pests/cis/2002/cis1302.htm). The data also showed that, at one location, a variety having a higher endotoxin concentration than another may actually have a lower concentration at a different location.
- Differences in susceptibility can also occur as a function of the geographic location of the population. Even before the commercialization of biotech cotton, it was well established that high temperatures can cause physiological imbalances in the plant that can trigger the degradation of soluble proteins. Consequently, concentrations of Bt toxins in any given variety may diminish if hot temperatures prevail for a long time. Chen *et al*. (2003, 2005) showed how exposure to temperatures of over 37º C for a 24-hour period reduces concentrations of Cry1A proteins by more than 50%. Geographical effects are pronounced due to various abiotic conditions that include not only high temperatures but also drought, salinity, water logging, etc. A study carried out by the Institute of Plant Protection of the Chinese Academy of Agricultural Sciences, China, showed that the toxin content in Bt cotton varieties changed significantly over time, depending on the part of the plant, the growth stage and the variety. Generally, the toxin protein was expressed at high levels during the early stages of growth, declined in mid-season, and rebounded late in the season.
- Plants contain many secondary compounds such as phenols, orthoquinones, terpenoids and tannins. Studies have shown that the concentrations of these compounds vary according to the age of the plant and exposure to external factors. Some of these compounds create synergies with Bt toxins (gossypol), while others (tannins) create negative interference. Kranthi *et al*. (2005) reported that, in times of stress, the relative increase in the concentration of gossypol makes up for the reduced concentration of Bt toxin. These findings show that changes in efficacy do not depend exclusively on the level of Bt toxin in the plant, but also on the plant's physiological condition. Similar lines of study on transgenic potatoes found that foliar glycoalkaloids in transgenic potato varieties affected the anti- insect benefits of the transgenes. This kind of research has not been done on cotton but it may probably be required. The conclusions of such studies might even help to enhance the plant's ability to produce greater amounts of toxins.
- Insect exposure to the same chemicals over a long period of time (i.e., frequent applications of the same insecticide year after year) enables the insect to develop the capacity to tolerate insecticide concentrations considerably greater than the recommended doses. Sucking, as well as chewing insects, are equally capable of developing such tolerances. Lepidopterans, for example, have developed resistance to insecticides in many countries and it is generally admitted that target insects can develop resistance to Bt toxins; in fact, resistance to the Bollgard Cry1Ac gene has been confirmed in many countries. Once a target insect develops resistance, the variations and fluctuations of endotoxin concentrations resulting from the many factors mentioned above will require greater numbers of insecticide applications, even on dual-gene biotech cotton.

New Insect-resistant Bt Based Technologies

The two new insect-resistant technologies that are expected to become available for commercial use in the next few years are Bollgard III® and TwinLink™. Both technologies are pending regulatory registration and the appropriate approvals, but they are already undergoing extensive testing in Australia and the USA. Last year, Monsanto applied to the US Environmental Protection Agency for permission to carry out field tests of genetically engineered triple-gene insect-resistant Bollgard

III cotton. Bollgard III combines the established Bollgard II gene (MON15985), which produces Cry1Ac and Cry2Ab2 toxins, with Syngenta's COT102 (Vip3Aa19), for the control of lepidopteran insects. The primary objective of the new technologies in the new products that are expected to come on line in the near future is to prevent the development of resistance for as long as possible and increase the spectrum of lepidopteran insects effectively controlled by the toxins. Bollgard III is expected to provide better protection against fall armyworms. Some early trials conducted on non-Bt cotton, Bollgard II and Bollgard III indicated that fall armyworm larvae were capable of penetrating 47%, 18%, and 3% of bolls on non-Bt cotton, Bollgard II, and Bollgard III, respectively.

The TwinLink™ Bt technology will offer an alternative to existing Bt technologies. The TwinLink™ insect resistance technology contains two Cry genes, expressing Cry1Ab and Cry2Ae proteins targeting lepidopteran pests on cotton. A number of trials were conducted in 2010 to further characterize lepidopteran control, confirm agronomic performance, compare varietal background performance, and determine protein expression profiles. The findings showed that plots of non-Bt cotton suffered 100% boll damage from bollworms. On the same date, plots of protected TwinLink™ technology sustained less than 10% damage to bolls, while bollworms caused about 15% boll damage in unprotected TwinLink™ plots. Average seasonal boll damage was less than 10% and 20% in protected and unprotected plots of TwinLink™ cotton, respectively. Yields from protected and unprotected plots of TwinLink™ cotton were similar, indicating that performance was only minimally increased with supplemental control of bollworms.

In another study, Cry1Ab and Cry2Ae protein concentrations were determined by protein extraction from terminal leaf tissue and quantitative, colorimetric ELISA procedure. Five locations and multiple genetic backgrounds were sampled for six consecutive weeks during the flowering and boll set period. The data indicate that, as with other Cry1 proteins, a slight decline of the TwinLink™ Cry1Ab protein takes place as the cotton plant matures. However, the Cry2Ae protein in TwinLink™ either maintained, or numerically increased its expression level through to maturity. These data indicate that under certain conditions, including extreme lepidopteran pressure (Greene *el al*., 2011), supplemental lepidopteran control may be needed to bolster the efficacy of the TwinLink™ technology

The discussion of the data above ndicates that it is not only the existing insect-resistant biotech cottons that may require additional spraying; the new Bt technologies (Bollgard III and TwinLink™) that are in the pipeline for approval (and expected, hopefully, to be in use within the next 2-3 years) will also need insecticide applications to get maximum yields. However, it is equally true that this situation is also linked to insect pressure and, as such, may vary from year to year. Which gene is effective against which lepidopteran will always be a factor in determining the need for insecticide applications, and at what economic net returns.

Conclusion

Insecticides are sprayed on non-biotech cotton based on certain thresholds for various pests. Thresholds may be based on a combined level of assessment of various pests. The use of a combined threshold might prevent the losses from one pest from reaching too high a level before insecticide applications are made. When a threshold is verified on non-biotech cotton it generally means that at least some damage has already been sustained prior to the initiation of insecticide application. Conversely, with insect-resistant biotech cotton, there is no threshold for any target pest and there is 100% pest control under all circumstances. If Bt proteins are not 100% effective, as has been shown to be the case with Bollgard II and WideStrike, which allow some bollworm damage in the field, spraying of insecticides may increase yields. Damage and benefits vary from year to year depending on the level of pest pressure. Data and annual surveys have shown that in the USA, Bollgard II and WideStrike varieties benefit from a single application of insecticide against the bollworm. Bollworm treatment increased lint yields by an average of 78 kg/ha and 125 kg/ha respectively with Bollgard II and WideStrike across all varieties and years evaluated. When bollworm insecticide costs, estimated insect protection and seed technology fees were factored into the equation, Bollgard II and WideStrike varieties provided economic returns as expressed in terms of bollworm control. Furthermore, everything seems to indicate that the more effective technologies expected to come on line within the next 2-3 years may also provide economic returns as expressed in fewer insecticide applications.

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Cotton Color: Measurement and Discoloration

The two most used independent parameters of cotton color are: degree of reflectance (Rd) and yellowness (+b). Reflectance indicates how bright or dull the cotton is, and yellowness indicates the degree of color pigmentation. The color code is determined by locating the point at which the Rd and +b values intersect on the Nickerson-Hunter cotton colorimeter diagram for Upland cotton.

The amount of sunlight, day and night temperatures during growth, agronomic inputs and their time of application are responsible for most of the variations in fiber quality parameters within a single variety. Fiber color is also affected by the same factors. Cotton grade is a composite assessment of color, trash and preparation. Each feature is judged separately, but a qualified classer integrates his assessment of the three diverse parameters into a composite grade. Color assessment is a primary category used to assign a grade to cotton. Trash used to be the second most important factor in determining quality, but advances in equipment have made it increasingly easy to eliminate plant materials from cotton, thus underscoring the significance of color as an important quality factor. While measuring fiber quality parameters has improved a great deal, adding to the reliability and repeatability of data, color measurement has not achieved similar successes. Part of the problem is that human color perception results from the interaction of three components: a light source, an object, and a detector (the eye and brain in the case of manual grading). So, when the color of a sample is measured, it is in fact a process whereby color, as perceived by humans, is measured and described by the naked eye or by a color-measuring instrument (HVI, Colorimeter, etc.). This article deals with the latest developments in fiber color measurement and progressive changes in color due to various reasons.

Measuring Color

Cotton color can be judged visually with the naked eye or measured mechanically with the help of different kinds of instruments. Naked-eye assessment of color, often referred to as classer's color grade, can be affected by the light under which a cotton sample is observed and by the general surroundings (table color, wall color, etc.). If the color of a sample has to be judged visually by a classer, the lab must make certain that the proper lighting is provided. Humans classify samples by visual comparison with a set of physical standards under standard illumination. The assessment is made in a room with dark grey walls, and samples are placed on a black table with an incident light intensity of 1,200 lx. The more experienced the classer is in judging cotton color, the more precise the assessment he/she can make. Classers require special training before qualifying for the job of assessing cotton color.

The US Department of Agriculture (USDA) started developing instruments to assess cotton color in the 1930's. The two criteria for color measurement (Rd and +b) were introduced at the time. According to Matusiak and Walawska (2010), Nickerson and Hunter developed an objective method of measuring color using a Colorimeter in the early 1940's. The Hunter scale used in a Nickerson Hunter Cotton Colorimeter indicates the percentage of reflection (Rd) in a vertical direction, which is a measure of the lightness of a sample, and in a horizontal direction the color code is determined by locating the point at which the Rd and +b values intersect on the diagram. During a color test, photodiodes absorb filtered light from the illuminated sample, and a microprocessor expresses the results in terms of the lightness and yellowness of the sample. Matusiak and Walawska reported (2010) that colorimetry technology was incorporated into HVI testing