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APPLICATIONS OF MICROSCOPY IN THE PAPER INDUSTRY:
CASE HISTORIES OF THE MEAD CORPORATION

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Abstract

Microscopical applications in the Mead Corporation are herein discussed: 1) defining proper starch cook for maintaining paper quality, 2) microbial degradation of paperboard used for beverage cartons, and 3) examination of high oxygen barrier plastic cups for hermetic seal and barrier construction. Visualization of the cooked starch by iodine staining and polarized light (PL) microscopy is a quick diagnostic aid to Mead mills. Scanning electron microscopy (SEM) and particularly PL proved useful in examining fiber biodegradation by fungi on Coated Natural Kraft™ beverage cartons. Nomarski differential interference contrast (DIC), PL, and SEM aided in qualifying lid materials for Mead's Crosscheck® Food Packaging System, defining hermetic seals, and examining container construction. Further, DIC optics have enhanced the observation of possible abnormalities in container construction used on Crosscheck over conventional Köhler optics and/or SEM. Integrative microscopy was thus a valuable technique in problem resolution for the defined applications.

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Introduction

Microscopy is frequently utilized for a broad range of problem solving in the Mead Corporation. Three cases will be presented that represent typical problems encountered by the microscopist that may be assessed by integrative microscopy. 1) Starch used as internal sizing in papermaking will exhibit an altered morphology under improper cooking conditions, and this leads to poor paper quality. Polarized light (PL) microscopy and differential interference contrast (DIC) optics that permit quick diagnostic analyses of uncooked or crystallized starch are discussed. Also reviewed is traditional iodine staining followed by microscopical examination which serves as a quality control tool for determining amylose/amylopectin ratio as guaranteed by the manufacturer. 2) Observations on manufactured paperboard products subjected to bacterial or fungal activity has not been reported. The second case considers morphological changes observed by scanning electron microscopy (SEM) in beverage cartons manufactured by Mead that have been treated with microorganisms and held under laboratory conditions. 3) The last case discusses multi-layer high barrier cup construction and irregularities that may contribute to reduced oxygen barrier properties. Interest in high barrier plastic cup analysis began when packaged applesauce product occasionally grayed. Oxygen contamination was considered a possible cause for the problem. Also reviewed are optical techniques for evaluating the critical aseptic packaging parameter of hermetic seal.

The integrative techniques of SEM, PL microscopy, Köhler optics, and Nomarski DIC optics are compared for their advantages in visualizing morphological properties of starch, beverage cartons, and high barrier cups.

Methods and Materials

Integrative Microscopy

Polarized Light Microscopy A Leitz Ortholux II Pol-BK polarizing light microscope was used for polarized light microscopy. For discussion of the theory of PL microscopy the reader is referred to basic texts (McCrone et al, 1984).

Photomicrography was done using the Wild-Leitz MPS 46 Photoautomat automatic exposure 35 mm system. Kodak

Ektachrome 160 film was used for the original photomicrographs, and prints were made from selected slides.

Nomarski Differential Interference Contrast Microscopy
Olympus Vanox-S AH-2 was utilized for Nomarski DIC microscopy. Both epi-illuminated (xenon) and transmitted (tungsten) Nomarski DIC microscopy were possible on this microscope.

Differential interference contrast (DIC) after Nomarski is the method of choice when the specimen of interest has inherent low contrast. The principles of Nomarski DIC optics are less well known and will be described here (see Figure 1). A beam of light passes first through a polarizer, converting the beam to one which is plane-polarized which then passes through a modified Wollaston prism (Figure 2) that splits the beam slightly and focuses this split beam in the front focal plane of the condenser. The plane-polarized, split beam continues through the condenser, specimen, into the objective, and then into a second modified Wollaston prism. This second prism recombines the split beam. The single plane-polarized beam is then passed through a second polarizing filter, the analyzer, which converts it into a fully polarized beam. At this point, the image is ready to be observed or recorded.

A key point in Nomarski DIC optics is that the split of the single beam is so minute that it is below the resolution of the objective lens used. This means that the image obtained is a differential, or three-dimensional, image. It is a false 3D image, and care must be taken in any interpretation of three-dimensionality in a Nomarski image.

Scanning Electron Microscopy A JEOL T220A scanning electron microscope was used in these studies. Photomicrographs were taken at accelerating voltages of 15 or 25 kV and various magnifications.

Photomicrography Photomicrography was done using the Wild-Leitz MPS 46 Photoautomat automatic exposure 35 mm system. Kodak Ektachrome 160 film was used for the original photomicrographs, and prints were made from selected slides.

Starch Preparations

PL and Nomarski DIC microscopy were utilized in studies on various laboratory and production starch solutions. The excellent definition of starch granules by PL microscopy was shown to be instrumental in determining the difference between properly cooked and uncooked starch solutions. In the laboratory, a solution of cornstarch was photographed in polarized light before and after boiling for 2 hours. DIC optics were utilized to increase contrast in production samples of a potato starch used as a sizing agent. DIC optics were also used to analyze mill starch sizing solutions for type II amylose and uncooked starch grains.

Beverage Carton Study

Fungal Preparation The fungal preparative procedure used was a modification of the Technical Association for the Pulp and Paper Industry (TAPPI) Procedure T-487 pm-85 entitled "Fungus Resistance of Paper and Paperboard" (1989). *Verticillium* and *Bortyris* or *Penicillium* from in-house cultures were subcultured on Potato Dextrose Agar for 4 days and harvested with 5 ml sterile water.

Beverage Board Preparation Beverage carrier boards were cut into 50 mm x 50 mm squares using flame-sterilized scissors.

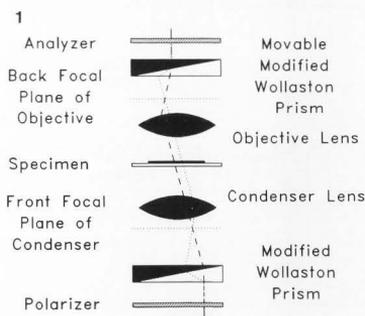


Figure 1. Schematic of a Nomarski differential interference contrast microscope.

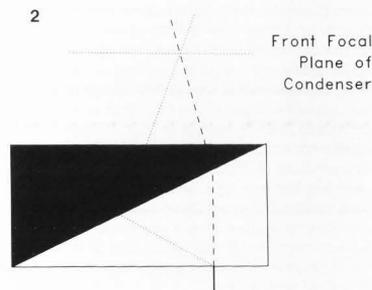


Figure 2. Schematic of a Modified Wollaston prism.

The pieces were taken from the centers of the boards to avoid tainted outside edges, and their surfaces were exposed to UV-C (germicidal) light for 30 seconds to reduce surface contamination. The pieces were then placed on prepared Petri dishes containing sterile mineral salt agar with 0.5% dextrose added. Agar formulation (TAPPI, 1989) is as follows:

Agar	10.0 g
Ammonium nitrate (NH ₄ NO ₃)	3.0 g
Dipotassium phosphate (K ₂ HPO ₄)	1.4 g
Magnesium sulfate (MgSO ₄ ·7H ₂ O)	0.25 g
Potassium chloride (KCl)	0.25 g
Tap water	1000.0 ml

Beverage Board Inoculation Two-tenths ml of the fungal inoculum was pipetted onto the surfaces of the boards, spread with a sterile glass rod, and placed in the incubator at 25°C for up to 6 weeks. Culture sources were from stock of environmentally contaminated Coated Natural Kraft cartons.

Control boards were cut into squares as described above and

placed onto sterile mineral salt agar. These boards were also held for up to 6 weeks. Subsequent microscopy revealed growth of native bacterial microflora; boards were not inoculated.

Preparative Microscopy on Boards Small corner pieces of board were removed and immediately immersed in vials containing chilled 2% glutaraldehyde fixative. These capped vials were then washed with distilled water 3 times over a half hour period and were post-fixed in a 1% solution of osmium tetroxide for a minimum of two hours. Some samples were allowed to sit for longer periods of time because they were judged not to be adequately fixed due to their light color. Note that all solutions were kept cold (4° C) until the 100% ethanol step. After this point, all solutions were used at room temperature.

Following adequate fixation and rinsing, the specimens were dehydrated through a graded series of ethanol solutions, 10 min in each change, up to 95%. They were put through two changes of 100% ethanol as a final dehydrant. At this point, the samples were split into two groups: one group was embedded for cross-sectioning and optical microscopy; the second was mounted on stubs for SEM.

The group to be embedded was placed in a 1:1 mixture of 100% ethanol and LR White embedding media (an acrylic resin). This was allowed to infiltrate for 1/2 hr. The specimens were then allowed to infiltrate for the same time period in 100% LR White resin. Following these infiltration steps, the specimens were embedded overnight in fresh 100% resin in a 60° C oven.

The samples to be used for SEM were taken from 100% ethanol and put through two changes of pure Freon™. They were then critical point dried using liquid CO₂ as the drying fluid. The specimens were mounted on stubs with double stick tape, given a thin coat of gold/palladium for conductivity and observed in the SEM. Magnifications are as noted. These procedures are modifications of methods outlined in Hayat (1989).

High Barrier Plastic Cups

Cups examined were manufactured by Rampart Packaging, James River Corporation, and Ball Corporation. Their general construction was polypropylene/adhesive/ethylene vinyl alcohol(EVOH)/adhesive/polypropylene; some cups were clear while others were opaque and contained titanium dioxide. End-use is in primary food packaging (e.g. Rampart cups are recommended for use in the Mead Packaging Crosscheck® High Acid Aseptic Packaging System).

Seal development was viewed by cutting out a portion of the flange and adjoining foil lid area and observing under Köhler and Nomarski DIC optics. Cup structure was examined by cross-sectioning container walls and observing the samples using Köhler and Nomarski optical microscopy. In order to improve visualization of the cup layers, portions of cup walls were embedded in BEEM capsules with LR White acrylic resin. Capsules were placed in a 60° C oven overnight. Blocks were trimmed and 8 µm sections were cut on a sliding microtome. Sections were mounted on glass slides and photographed under various optical conditions.

Results and Discussion

The areas discussed below demonstrate the utility of using integrative microscopical approaches to solve product and process problems in the industrial laboratory.

Starch Preparations

Differential iodine staining provides a quick, reliable method for the quantification of amylose and amylopectin fractions of a starch suspension. Grains which are primarily amylose (linear polymer) will stain blue, while those which contain mostly amylopectin (branched polymer) absorb less stain and appear red (Figure 3) (Whistler, 1965, 1984; Young, 1984). It has been shown that it is the amylose fraction which binds iodine to give the blue color (Hollo & Szeitli, 1968). This simple microscopical test allows an approximate calculation of the amylose to amylopectin grain ratio in any starch suspension. Hixon and Brimhall (1968) point out that iodine staining is a good method of differentiating between waxy (red-staining, amylopectin) and non-waxy (blue-staining, amylose) corn starch. Thus, there is applicability in the food industry as well as in the paper industry.

One problem often encountered in the paper industry is that of improperly cooked starch for sizing applications. At one of Mead's mills, starch solutions are monitored on a weekly basis for uncooked starch grains. The routine method for this procedure is PL microscopy. When viewed under fully crossed polars, starch exhibits the familiar polarization or Maltese crosses (Figure 4)(Fitt & Snyder, 1984; Moss, 1976; French 1984; McCrone & Delly, 1973; Wurzburg, 1986). Proper cookout of starch solutions should eventually burst most of the grains (Mahrer & Cremer, 1986). As the starch grains swell upon heating, they gradually lose their polarization crosses (Figure 5)(Sterling, 1974; Yahl, 1984). When samples from the mills are examined under fully crossed polars, no polarization crosses should be present: all grains should have been burst by the heating process. Any polarization crosses which remain indicate improper cookout. If a large number of grains show polarization crosses, this is cause for concern by mill engineers. Thus, PL microscopy may be employed as an excellent quality assurance procedure for monitoring internal sizing.

Another potential problem that is monitored by PL microscopy is the formation of Type II amylose. Through a process called "fractionation" (Mahrer & Cremer, 1986; Young, 1984; Whistler, 1965), starch solutions can crystallize. Small (Type I) amylose crystals can form from the linear polymer, with no ill effects, if the starch is allowed to cool to 153-193° F. If held in this range for any appreciable length of time, the large Type II amylose crystals can form (Figure 6)(Mahrer & Cremer, 1986, Figure 6, p. 225). Type II crystals can cause rejection and slinging at the nip of the size press and can puncture the paper as it passes through the nip.

A final technique utilized often at The Mead Corporation is that of Nomarski differential interference contrast (DIC) microscopy of starch. Nomarski DIC optics were found to be ideal for detailed observations of cooked mill starch solutions. Industrial starch grains at complete cooking show much more detail under Nomarski optics than under normal Köhler optics. Figures 7A and B show solutions that have been cooked, and the

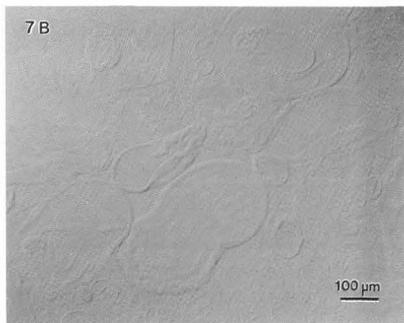
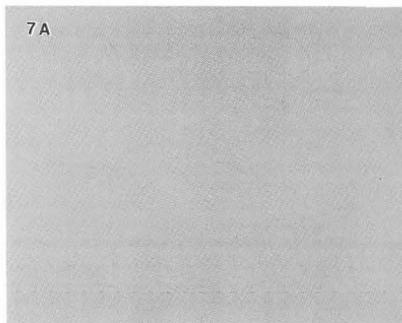
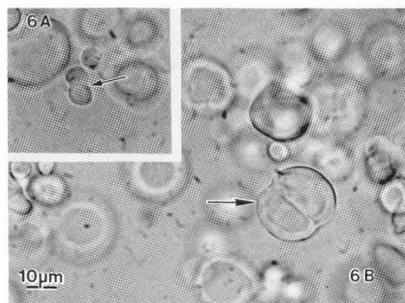
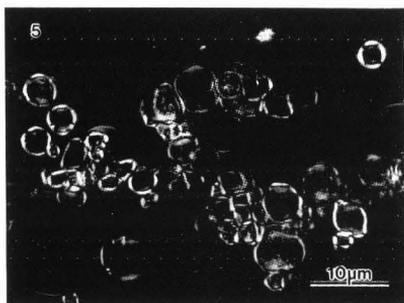
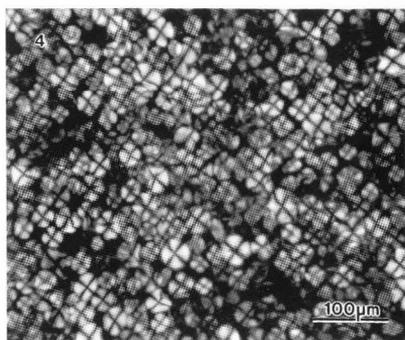
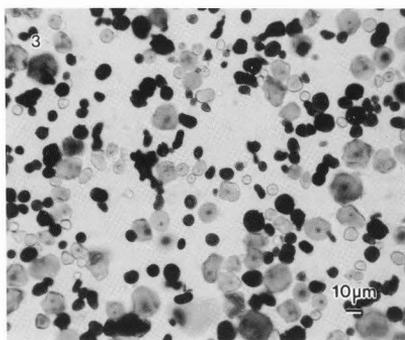


Figure 8. Using SEM, hyphae were seen penetrating pits (8A, arrow) of coated board as well as growing inside fibers (8A and B, arrowheads).



grains are very swollen and near bursting. Under Köhler optics (Figure 7A), they exhibit extremely low contrast. When viewed under DIC optics, however, grain detail is heightened considerably (Figure 7B) and cook-out can be definitively assessed.

Beverage Carton Study

Integration of microscopical techniques proved extremely useful in the analysis of laboratory-induced biodegradation of Mead beverage cartons. It is well documented that both fungi and bacteria attack and degrade cellulosic materials (Allsopp and Seal, 1986). So-called soft-rot, white-rot, and brown-rot fungi (Kirk and Cowling, 1984) as well as certain bacterial species (Boyce, 1961a) contain enzyme systems which allow them to degrade cellulosic materials. Indeed, of major concern today is the development of plastics which are degradable by fungi and bacteria (Studt, 1990). In the present study, our investigations assessed fungal-inoculated paperboard incubated under laboratory conditions and bacterial degradation of paperboard by native bacterial microflora. It was concluded that manufactured wood-based products degrade similarly to wood in the native state, and this activity can be qualitatively monitored by PL microscopy as discussed below.

Many microscopical techniques have been used in the literature to study bacterial and fungal degradation of wood and wood products. Several authors have shown the usefulness of the transmission electron microscope (TEM) in bacterial degradation of fibers of both softwood and hardwood species (Singh, 1989; Singh et al., 1987). Of particular interest were micrographs that show tunnels formed by bacteria through the wood cell wall. TEM of fungal decay has also been done (Chou and Levi, 1971; Kollmann and Côté, 1968). Many authors have made use

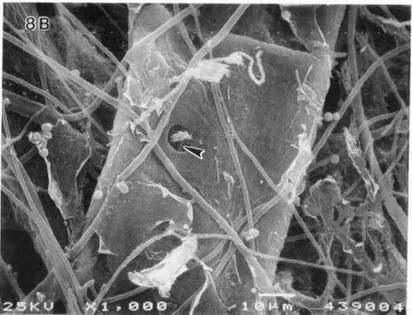


Figure 3. Iodine staining is a good method for differentiation of amylose from amylopectin. In this optical photomicrograph, the linear polymer amylose stained blue. The branched polymer absorbed far less iodine and stained a reddish color.

Figure 4. Starch viewed under fully crossed polars exhibits familiar Maltese crosses.

Figure 5. Solution of cornstarch that was boiled for 2 hours. Note the partial loss of polarization crosses under fully crossed polars.

Figure 6. Small (Type I) amylose crystals can form from cooked starch as it cools (6A, arrow). Large (Type II) crystals can form when cooked starch is held between 153° and 193° F (6B).

Figures 7A and B. The potato starch sample, after it is properly cooked, is very difficult to image due to its inherent low contrast. In 7A the grains are barely visible, but under DIC optics the grains are very distinct (7B).

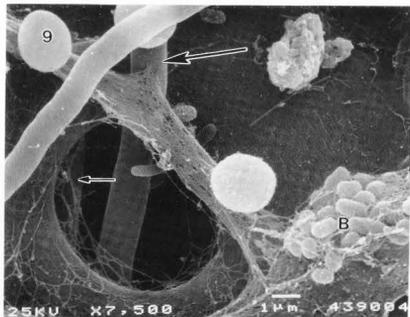


Figure 9. Bacterial growth (B) on fibers in coated board is shown in this SEM photomicrograph and their meshwork surrounds the pit. A fungal hypha (arrow) entered the pit, while another (small arrow) was seen inside the fiber.

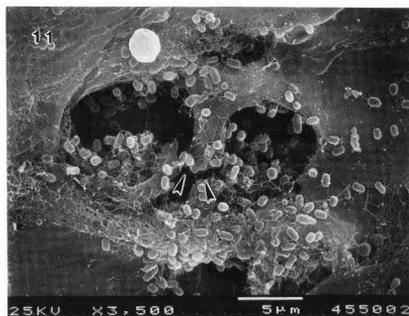


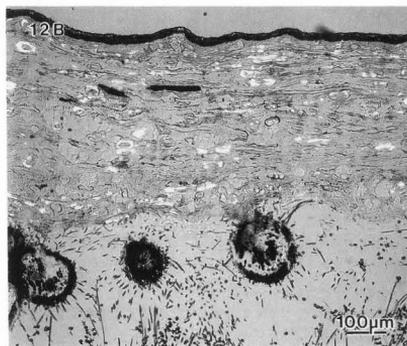
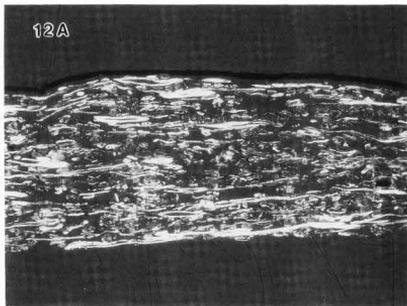
Figure 10. Hyphal growth has penetrated the coated side of the board, creating decay troughs (arrows) and holes.

Figure 11. The SEM photomicrograph shows damage of fiber pits in coated board by bacteria including breaks in the walls of the pits (arrowheads).

Figure 12. PL microscopy of a coated board cross-section shows high birefringence of fibers in the control (A) but reduced birefringence (i.e. loss of fiber integrity) after 6 weeks of fungal growth (B).

of the SEM in examining bacterial decay (Levy, 1975; Kirk, 1983) as well as in the studies of fungal decay (Parham, 1983; Eriksson et al., 1980). SEM and optical microscopy were readily applicable in the present work to assess characteristic growth patterns of both bacteria and fungi.

In the six week non-inoculated specimens, hyphal growth penetrated through pits and inside fibers (Figures 8 and 9) and the coating (Figure 10) creating decay troughs. Note also the outgrowth of native bacteria and their fine meshwork on the fibers (Figure 9B). These characteristic growth patterns have all been shown in the literature for wood (Eriksson et al, 1980;



Parham, 1983). Results of the present studies showed bacterial and fungal degradation to occur on Mead Coated Natural Kraft thus documenting that manufactured wood-based products can react similarly to native wood in the presence of microorganisms.

Certainly a useful technique for studies of this type is that of optical microscopy although the SEM advantage of dimensionality is lost. The brightfield microscope has proved useful in showing microbial decay of cell walls (Schmidt, 1978). A complete summary of anatomical changes associated with bacterial and fungal decay of wood has been published (Wilcox, 1970). The optical microscope was instrumental in this anatomical analysis. Brightfield, darkfield, PL, and phase contrast microscopies were used to study patterns of bacterial decay in wood (Greaves, 1969). Bacteria have been shown to attack pits and bordered pit membranes (Levy, 1975). In studies using brightfield microscopy by Proctor (1941), Butcher (1975), Boyce (1961b), and Panshin and DeZeeuw (1980), photomicrographs illustrating hyphal penetration of wood cell walls are presented. In the present study similar growth patterns were observed.

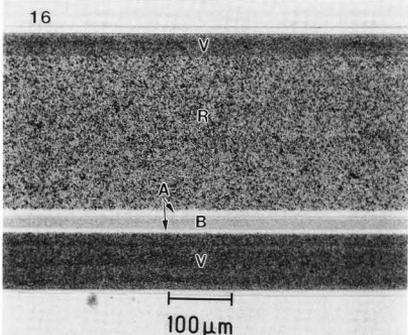
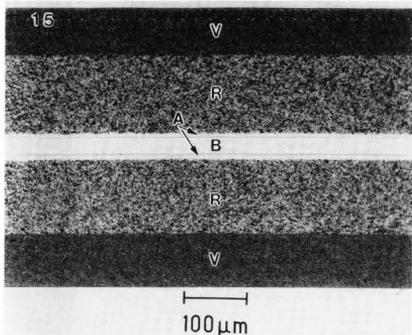
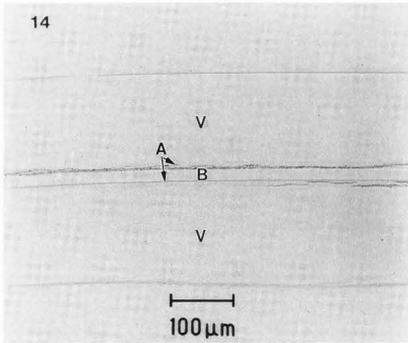
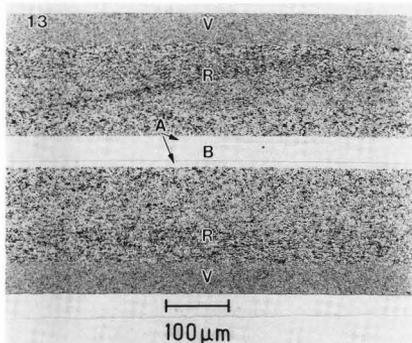


Figure 11 shows fiber pits enlarged by outgrowth of native bacteria and breaks in the walls of the pits.

Perhaps the most interesting optical microscopy technique applied in this study was that of PL microscopy. Under PL microscopy fibers exhibit birefringence - a property of most crystalline substances. This property is well documented in the literature (Wilcox, 1965 and 1968; Greaves, 1969; Fengel and Wegener, 1984; Liese, 1970.) In any crystalline particle or fiber, if the crystalline state is disturbed or lost, so is the phenomenon of birefringence. This fact was instrumental in showing that bacteria and fungi do degrade wood fiber integrity (Figures 12A and B). At present there is no commonly accepted index of biodegradation. The studies herein reported are indicative of the potential for the use of birefringence as a qualitative index of degradation in environmental testing of wood-based products.

High Barrier Plastic Cups

Microscopy enhances observations on cup construction and is essential for detailed observations. Cup cross-sections of polypropylene (both virgin and regrind), adhesive layers, and

Figures 13-16. Köhler optical cross-sections of Rampart titanium-tinted (13) and clear (14), Ball (15), and James River (16) high barrier cup walls show virgin polypropylene (V), regrind (R), adhesive (A), and barrier (B) layers. Note that the clear Rampart cup has no regrind and the James River cup has an asymmetrical structure with the barrier being closer to the outside of the cup wall. In addition the barrier of the James River cup appears to be of a different composition from the rest.

EVOH layers can be seen with SEM, but observations can be made using optical microscopy. The differences among the constructions of the two Rampart cups (titanium-tinted and clear)(Figures 13 and 14), the Ball cup (Figure 15), and the James River cup (Figure 16) can be clearly seen in cup wall cross-sections. Titanium-tinted Rampart cups have the most regrind - a reflection of production demand. The clear Rampart cup has no regrind (Figure 14) for optimal clarity. Variations in wall thicknesses among the samples are a result of customer specification and are not a function of the manufacturing process. Comparing Figures 13, 14, and 15 with Figure 16, it may

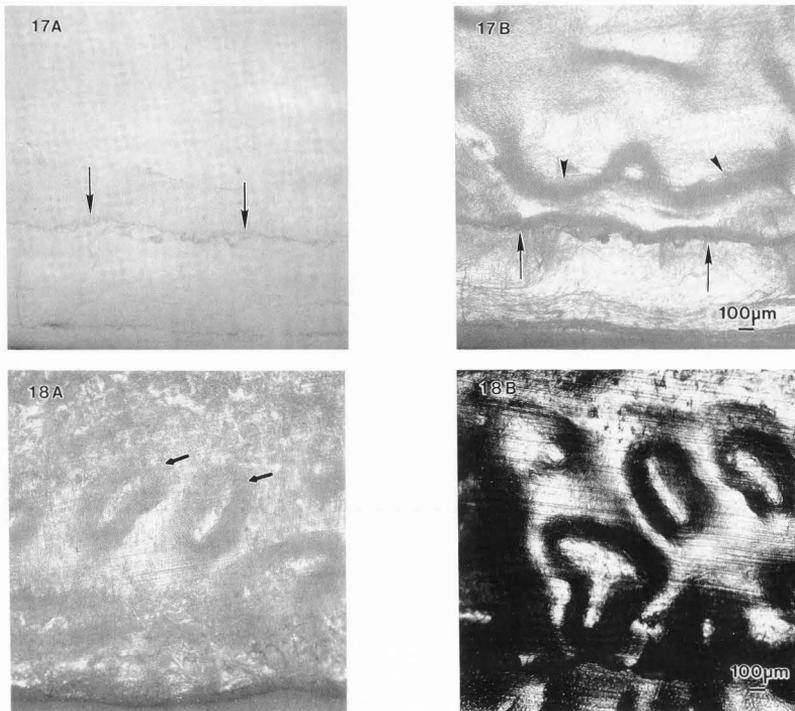


Figure 17. This figure illustrates the usefulness of Nomarski optics in observing cup seals. In normal brightfield microscopy, very little detail is observed (17A). The demarcation which indicates the edge of the seal is seen (arrows), but little other detail is noted. In DIC optics, more detail is evident (17B) such that the edge of the seal is now very obvious (arrows). Also, the pattern formed by the seal is visible (small arrowheads).

Figure 18. Using DIC optics, seal patterns of a cup flange (18A) and lid (18B) sealed with a Morprime sealant were observed. Notice the transfer of the lid embossing pattern to the seal pattern on the flange (arrows).

be seen that Ball and Rampart cups have the same oxygen barrier: EVOH. The James River cup appears to have a different composition of EVOH from the other cups. The barrier position of the James River cup is a special construction for retorting. Such a position permits wicking out of moisture through the moisture sensitive barrier by mass action thus prolonging the oxygen barrier property of the material.

Nomarski DIC optics frequently offered an advantage over Köhler optics by providing a rapid and efficient procedure for distinguishing hard to visualize seal development areas of the cups. Figures 17 A and B compare Köhler and Nomarski microscopy on the clear Rampart cup sealed with a Morprime® lid. As illustrated it is quite difficult to see definition of the seal area on the flange using Köhler illumination. Using Nomarski optics seal development of the high barrier cups is much easier to assess. Important parameters in Morprime-sealed cups are the width of the seal, signs of oversealing/flange deformation, and transfer of the embossing pattern on the lid/sealant interface to the flange (Figure 18A). The underside of the lid appears as a smooth surface with Köhler optics, but a pattern can be distinguished with Nomarski (Figure 18B). Seal parameters of coextruded lids are lack of sealant residue on the flange but evidence of good seal width as judged by pressure in the area applied by the seal head and lack of stringing (Figures 19A and B). Stringing can occur in Morprime-sealed lids, but these are less temperature sensitive than the coextruded lids which tend

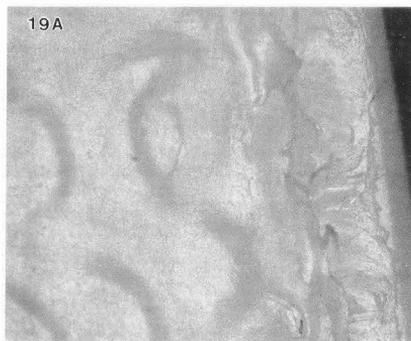


Figure 19. Using DIC optics, flange (19A) and lid (19B) seal activation patterns were visible on a cup sealed with a coextruded film type sealant.

to heat easily and release sealant and/or polypropylene in fine strands (Figure 20).

Morprime sealant fractures upon release of the lid such that some sealant transfers to the cup flange and remaining sealant stays on the lid (Figures 18A and B). The embossing pattern of the lid is also transferred to the sealant on the flange such that it may be visualized by the unaided eye but more easily studied by Köhler optics. Nomarski optics are not necessary for this application but are required to clearly observe the low contrast seal pattern for coextruded lids (not illustrated).

Intense examination of cross-sections was prompted when the user began experiencing graying of natural applesauce food product that had no oxygen scavengers. The problem led to unpublished SEM work in our laboratory which showed gaps or holes between the adhesive layers and the EVOH and between the adhesive layers and the regrind in all three types of cups.

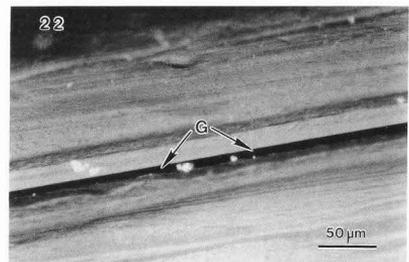
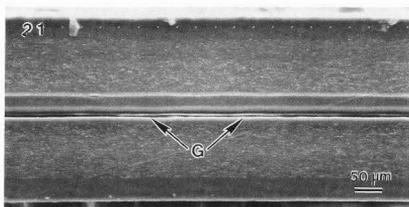
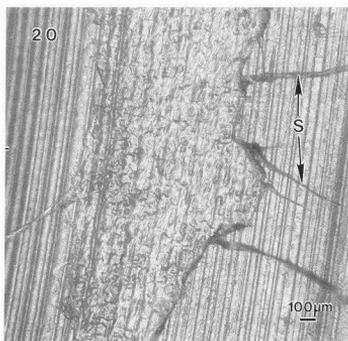


Figure 20. Cup flange with low contrast seal stringing (S) associated with coextruded film type sealant is shown using Nomarski optics.

Figure 21. The SEM photomicrograph shows a gap (G) in the layers of a high barrier cup. Most gaps were found between the barrier and adhesive layers. See optical photomicrograph, Fig. 22.

Figure 22. Gaps are still seen after cross-sectioning cup wall and observing using Nomarski optics (G). By using Nomarski optics the possibility of the gap being an artifact of SEM preparation was eliminated.

Figure 21 illustrates the gap in the Rampart cup. Rampart rawstock prior to cup formation did not show gaps. A problem in interpretation was the fact that in preparing the cup cross-sections for SEM the cup cross-sections were twice subjected to vacuum: sputter coating with gold/palladium and the required vacuum for observation in the SEM. To precisely interpret the true or artifactual presence of gaps, samples were embedded, sectioned, and viewed using epi-illuminated DIC optics. The images aided in discerning the actual presence of the gaps as seen in Figure 22. A short article in *Plastics World* (Callari, 1990) confirmed that gaps are real not artifactual and are a problem in production, but the problem is being addressed. Gaps or any flaws are of concern in high barrier cups for use in extended shelf life packaging of food because of the possible effect on oxygen and moisture transmission. The gaps present in the barrier structure may have contributed to the graying of applesauce although gaps were probably not the sole source.

Conclusions

Results of this study demonstrated that integrative microscopy is an excellent tool to interpret a broad range of problems within the Mead Corporation including cooking of starch, beverage carton degradation, and high barrier cup structural characteristics and sealing performance. In the cooked starch examination PL microscopy was adequate, but DIC optics were employed for best contrast and therefore most intense observation of the starch grains. For observing degraded board Köhler optics were quite adequate. However the phenomenon of birefringence in PL microscopy provided qualification of the degree of fiber degradation. High barrier cups were observed in cross-section by either Köhler or SEM microscopy, but for some applications such as seal observation, coextruded lid sealing patterns, and dimensional imaging with no vacuum DIC optical microscopy was the best choice.

Trademarks

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.