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# Use of Microscopy To Assess Bran Removal Patterns in Milled Rice

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**ABSTRACT:** During rice milling, the bran and germ are successively removed from the caryopsis (kernel). Because bran and germ contain large quantities of lipid, the amount of lipid remaining on the kernel surface may be used as a method for the assessment of milling quality. Bulk samples of rice pureline varieties and an experimental hybrid were milled for 0, 10, 20, 30, and 40 s. Scanning electron microscopy (SEM) revealed that brown rice kernels had large contours of linear protuberances and depressions running lengthwise along the kernel surface. The protuberances were abraded successively during milling, but varying amounts of material remained in the depressions. Light microscopy combined with the lipid-specific probes Nile Blue A or Sudan Black B demonstrated that the material in the depressions observed with SEM was lipid. Sections of whole, milled rice kernels, prepared using a modified sectioning technique and stained with Nile Blue A, showed that portions of the embryo remain after milling and that lipid is located on or near the surface of the kernel. Differences in quantity and distribution of residual lipid as milling duration increased were documented photographically to indicate the extent to which the bran and embryo components were removed during milling. This paper provides proof of concept that residual lipid is a robust measure of the degree of milling.

**KEYWORDS:** *Microscopy, rice milling, lipid localization, lipid staining*

## ■ INTRODUCTION

Degree of milling (DOM) refers to the extent of bran and germ removal from brown rice.[1−3] Bran that remains on the surface of the rice kernel affects the value, quality, stability, appearance, and functionality of the milled rice.

Rice, like most other cereals, has a fairly low lipid content on a whole kernel basis.[4−6] Generally, the majority of cereal lipids are found in the aleurone cells and embryonic tissues.[7] Rice milling involves removing much of the bran and germ by abrasion and results in a product that is light in color and has a longer shelf life compared to that of brown rice. White rice requires less time and energy to cook than brown rice and has specific, desirable functional characteristics.

How well rice is milled or DOM depends upon maximizing the milling yield by cleanly removing the bran and germ while minimizing removal of the starchy endosperm. Although there are different ways to assess DOM, the official standard measure of rice DOM is a subjective, visual method used by the Federal Grain Inspection Service, where test samples are compared to standard check or line samples.[8] Microscopy combined with specific staining for the residual components is a direct indication of DOM because the remaining tissue components are easily visualized. Because the outer tissues are high in lipid content, quantitative, chemical techniques that take advantage of the residual lipid, such as the measurement of surface lipid content,[9,10] give a good indication of DOM of the milled rice. The bran or lipid remaining on the surface of a kernel is not evenly distributed over the kernel. Therefore, an understanding of the anatomy of the rice kernel[11−14] and where the residual material occurs is important in refining methods of determining DOM, which could, in turn, increase the milling yield or possibly predict and/or explain milled rice functionality.

DOM is directly related to kernel anatomy because milling removes the outside layer of the kernel where the tissues are high in lipid and other components. For example, with scanning electron microscopy (SEM), Watson et al.[15] observed that one side of brown rice kernels contained three layers of aleurone cells, while the other side had only one or two layers. Thus, a mill could remove more of the aleurone on one side of kernels than the other, thereby impacting DOM.

Low-magnification light microscopy can be used to study individual kernels in various stages of milling. SEM is an excellent tool for observation of the exterior of entire milled rice kernels. In addition, virtually no modification of the sample is needed for sample preparation because the kernels are relatively dry and can withstand the high vacuum requirements of SEM. However, SEM has several disadvantages, including the expense of the equipment, the limited sample throughput, and the lack of specific staining techniques. Only the roughness of kernels can be determined with SEM, whereas chemical entities, such as lipids, carbohydrates, and proteins, may be identified and located using light microscopy combined with specific stains.

SEM has been used to relate DOM to the kernel structure;[15] however, this study was based on the amount of tissue disruption and not on a tissue component, such as lipid. In the present study, to determine where on the kernel the bran and germ remained after milling, intact individual kernels were observed using SEM before and after milling. Additionally, brown and milled rice kernels were left intact or sectioned, and both were stained for lipid. The intact kernels gave an indication of the amount of surface lipid remaining after milling. Sectioned kernels were used to ascertain the thickness

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Figure 1. Collection of intact rice kernel sections with Scotch packaging tape. Leica RM2265 rotary microtome with paraffin block containing rice kernels clamped in the microtome chuck (a). Collecting sequential, longitudinal sections of rice kernels (b and c). Slide showing two stained sections collected from a paraffin block containing 10 whole, milled rice kernels (d).

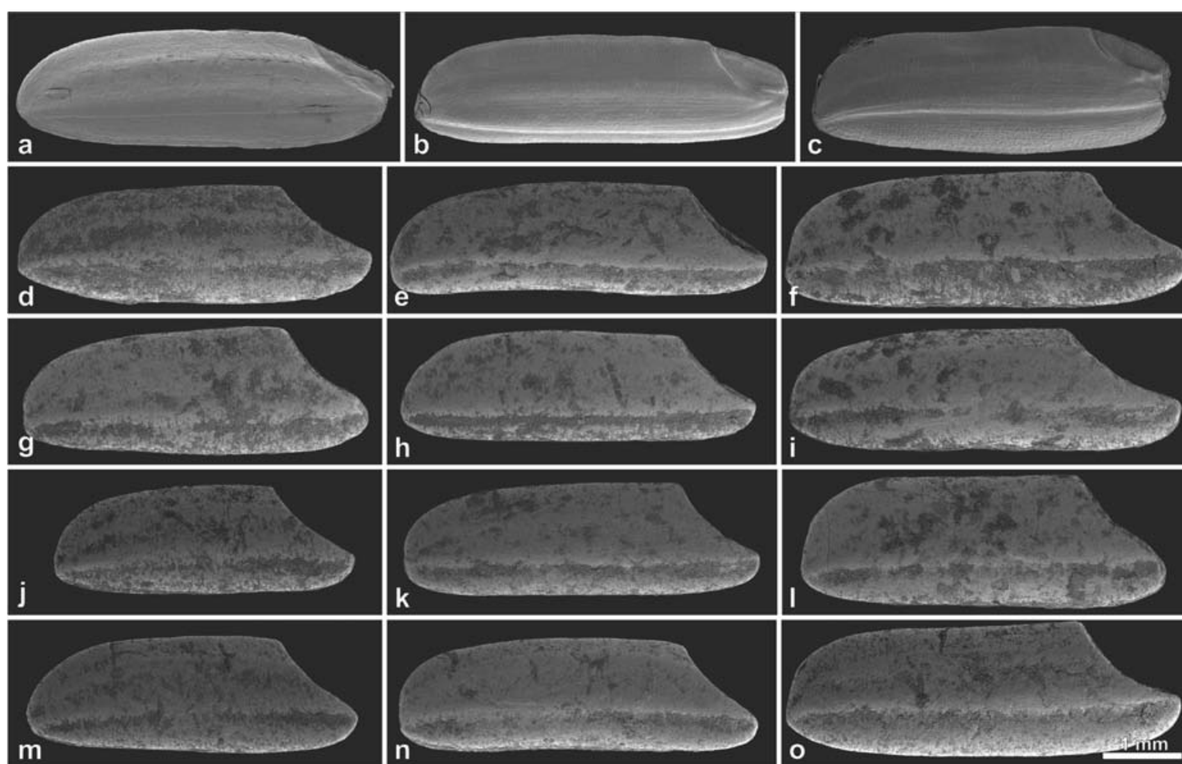

Figure 2. Scanning electron micrographs of whole and milled kernels of rice. Brown rice kernels (a–c). Rice kernels milled for 10 s (d–f), 20 s (g–i), 30 s (j–l), and 40 s (m–o). The rice cultivars are Cypress (a, d, g, j, and m), Wells (b, e, h, k, and n), and an experimental hybrid (c, f, i, l, and o). The growing locations are Corning, AR (a, b, d, e, and g), Newport, AR (h, k, and n), and Stuttgart, AR (c, f, i, j, l, m, and o).

of the remaining lipid residues or, by association, the degree of bran removal. Because lipids are extracted by the solvents used during standard embedding methods for microscopy, we used a modified method of paraffin encasement of the intact kernels[16] without prior fixation and dehydration.

## MATERIALS AND METHODS

**Rice Samples.** Rice cultivars Cypress, Wells, and an experimental hybrid were grown and harvested in 2008 in three locations in Arkansas (Corning, Newport, and Stuttgart). Each cultivar lot was dried to 12.5% moisture content and cleaned of material other than grain. A total of 100 samples (50 g) of rough rice were dehulled to produce brown rice. The brown rice samples were left unmilled or were milled for 10, 20, 30, and 40 s using a laboratory mill (McGill No. 2, Rapsco, Brookshire, TX).

**SEM.** Three representative intact kernels from each cultivar/milling set were mounted on aluminum specimen stubs using carbon adhesive tabs (Ted Pella, Inc., Redding, CA). The samples were coated with 3–5 nm of gold–palladium in a Denton Desk II sputter coating unit (Denton Vacuum, LLC, Moorestown, NJ) and imaged in a Hitachi S4700 field emission SEM (Hitachi High Technologies America, Inc., Pleasanton, CA). One representative image of each cultivar/milling set was captured at 1280 × 960 pixel resolution.

**Light Microscopy: Staining of Whole, Intact Kernels.** Representative whole, intact kernels were immersed in 0.01% aqueous Nile Blue A (CAS number 3625-57-8, Sigma-Aldrich Co., LLC, St. Louis, MO) for 30 s, rinsed briefly in water, and photographed while under water. Other representative whole, intact kernels were immersed in 0.3% Sudan Black B (CAS number 4197-25-5, Sigma-Aldrich Co., LLC, St. Louis, MO) in 70% ethanol for 10 min,[17] rinsed briefly in 70% ethanol, and photographed under 70% ethanol. The Sudan Black B in ethanol was stirred continuously for about 30 min because Sudan Black B is near its saturation point at 0.3% concentration. Three kernels from each cultivar/milling set were selected and viewed.

**Light Microscopy: Encasement for Sectioning.** The paraffin encasement and adhesive tape method of Ogawa et al.[16,18] was modified to accommodate a greater number of samples and used for

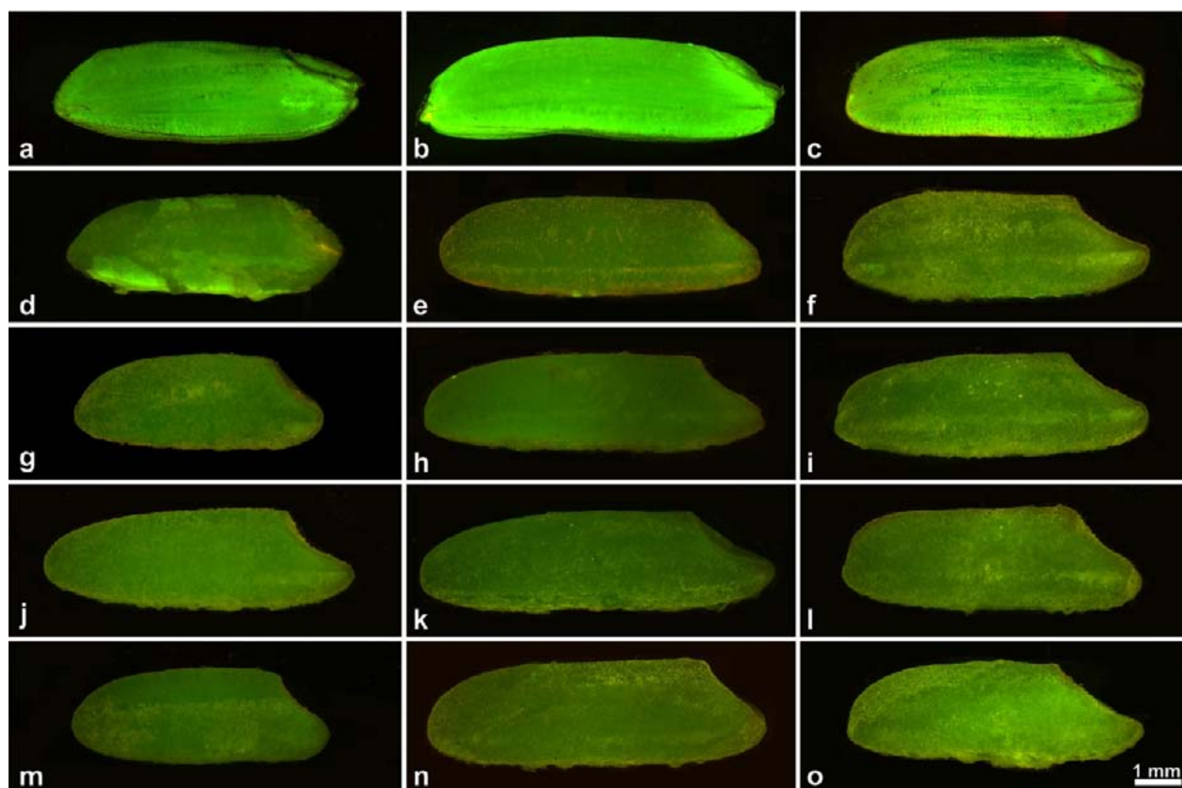


**Figure 3.** Fluorescence micrographs of intact, whole brown and milled kernels of rice stained with Nile Blue A for lipid. The kernels were stained intact and photographed while under water in a fluorescence microscope using a combination filter with an excitation of 450−490 nm and a barrier long pass of 515 nm. The yellow material is neutral lipid, and the bright green material is pericarp autofluorescence. Panels a−c show kernels with completely intact pericarp, and panel d has large pieces of remaining pericarp tissue. The darker green material is endosperm and probably results from autofluorescence of aromatic proteins and the phenolic compounds in the cell walls of the endosperm. Brown rice kernels (a−c). Rice kernels milled for 10 s (d−f), 20 s (g−i), 30 s (j−l), and 40 s (m−o). The rice cultivars are Cypress (a, d, g, j, and m), Wells (b, e, h, k, and n), and an experimental hybrid (c, f, i, l, and o). The growing locations are Corning, AR (a, b, d, e, and g), Newport, AR (h, k, and n), and Stuttgart, AR (c, f, i, j, l, m, and o).

sectioning. Dry rice kernels were encased in paraffin (i.e., paraffin was only used as a support and not as an infiltration medium) using a commercial tissue embedding system to melt and cool the paraffin (Leica EG1150H and C Modular Tissue Embedding Systems). Kernels from each cultivar/milling set were placed directly into embedding molds (Simport, Beloeil, Quebec, Canada) containing molten paraffin. Up to 10 kernels were placed in each mold. As the paraffin cooled, the rice kernels were oriented longitudinally. Before the paraffin was completely solidified, an embedding cassette (Simport, Beloeil, Quebec, Canada) was placed into the paraffin on top of the mold containing the samples. The paraffin was allowed to harden; the mold was removed; and the samples in the cassettes were stored at 4 °C until the blocks were sectioned. Two or more molds per cultivar/milling set were prepared, ensuring that a representative number of kernels were imaged.

**Light Microscopy: Sectioning.** The hardened block was faced on a rotary microtome (Leica RM2265) equipped with a disposable microtome blade (Leica 819 low profile) until near central longitudinal sections were visible. To collect intact sections, a strip of Scotch Brand Mailing and Storage tape (approximately 2 cm) was cut. The cut width needed to be about the same width as the paraffin block and less than the width of a standard microscope slide. The 5 cm wide roll of tape was an ideal length for collecting two or three sections per slide. One end of the tape was pressed firmly to the surface of the faced block, and a single 10 $\mu$m section was collected. A second section was then collected, adjacent to the first, and if a third section would fit onto the slide, a third section was collected (panels a−c of Figure 1). Once the sections were collected, the tape was affixed to a microscope slide using a thin strip of adhesive tape with sections facing up and then stained (Figure 1d). Sectioning, staining, and imaging of the sections was carried out on the same day for optimum lipid preservation.

**Light Microscopy: Staining of Sections and Photomicrography.** Slides were stained immediately prior to viewing. Water was placed under sections using a Pasteur pipet by first lifting the tape containing the sections and then lowering the tape again. Approximately 1 mL of 0.01% aqueous Nile Blue A[19] was evenly distributed on the surface of the section and topped with a cover glass. Whole, longitudinal sections were viewed and photographed using a Leica MZ16F stereo microscope equipped with an XCite EXFO fluorescence illumination source and a filter combination for fluorescence in the blue region of the spectrum (excitation, 450−490 nm; barrier long pass, 515 nm). Digital images were collected at 1600 × 1200 pixels using a digital camera (Retiga 2000 Fast Cooled 12-bit, Surrey, British Columbia, Canada).

## ■ RESULTS AND DISCUSSION

**SEM Observations of Rice Kernel Surfaces.** Although the surfaces of whole rice kernels were relatively smooth and consisted mostly of fine linear structures, they also had large contours of linear protuberances and depressions that ran lengthwise along the kernel (panels a−c of Figure 2). The dorsal side of each kernel had a distinctive embryo or germ. Some kernels exhibited fissures that were due to handling (Figure 2a). The protuberances were the areas of the kernels that were abraded first on initial milling, as shown in panels d−o of Figure 2. As milling proceeded, the protuberances were abraded. These samples were milled for different time periods

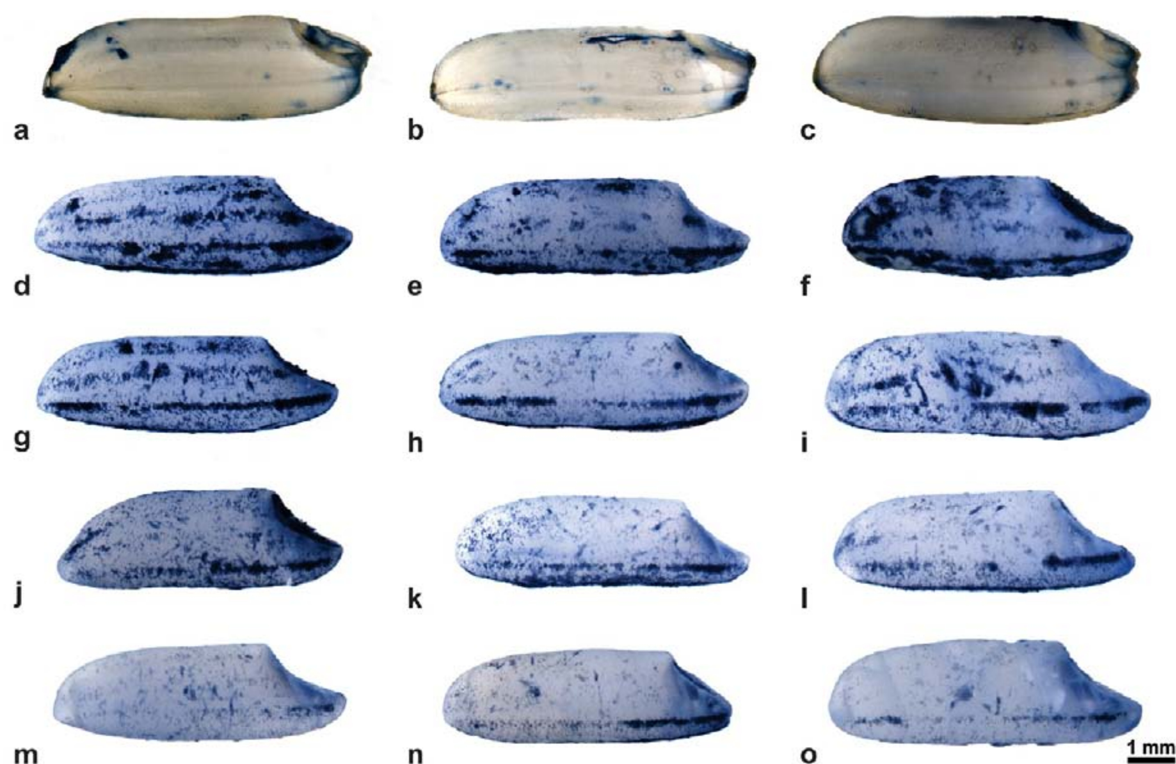


**Figure 4.** Brightfield micrographs of intact, whole and milled kernels of rice stained with Sudan Black B. Lipids are stained dark blue and indicate areas where bran remains after milling. The pericarp is unstained (panels a−c and circled red area in panel f). Staining occurs in a linear pattern in the longitudinal direction of the kernel because these are low-lying areas or depressions (stained, blue areas) inaccessible to the mill until the higher areas or protuberances have been removed (unstained, white areas). Brown rice kernels (a−c). Rice kernels milled for 10 s (d−f), 20 s (g−i), 30 s (j−l), and 40 s (m−o). The rice cultivars are Cypress (a, d, g, j, and m), Wells (b, e, h, k, and n), and the experimental hybrid (c, f, i, l, and o). The growing locations are Corning, AR (a, b, d, e, and g), Newport, AR (h, k, and n), and Stuttgart, AR (c, f, i, j, l, m, and o).

to illustrate the progression of milling over time with different cultivars. The embryo does not adhere tightly to the kernel, and the pericarp and aleurone layers that surround the embryo form a loose enclosure on its outer surface. Thus, most of the embryo was quickly removed during the initial stages of milling. However, bits of the embryo (i.e., the scutellum) still remained (Figure 2e), which were not always visible. This lack of visibility was because the exact orientation of the kernels could not be precisely controlled during preparation for microscopy and because the embryo is located in a concave area of the kernel. For this reason, the remaining embryo is only evident in Figure 2e.

**Light Microscopy of Rice Kernel Pericarp.** Brown rice kernels autofluoresced an intense green (panels a−c of Figure 3) using the blue filter combination (excitation, 450−490 nm; barrier long pass, 515 nm). The intense fluorescence is due to the presence of phenolic compounds in the pericarp.[20,21] Although the kernels were stained with Nile Blue A prior to viewing, the yellow fluorescence from the lipids was not evident because the dye could not penetrate the pericarp. Even if the dye were able to penetrate the pericarp, fluorescence from the stain would not be visible because the intense autofluorescence of the pericarp would dominate the signal from the Nile Blue A-stained lipids. Therefore, any bit of pericarp remaining on the surface of a kernel and covering most of the remaining embryo, as seen in Figure 3d, masked lipids below the surface. Most of the embryo is intact in this micrograph but is covered by pericarp. Consequently, the lipid in the embryo can only be seen in places where there was a fissure in the pericarp. Once the pericarp was removed, lipids on the kernel surface were visible (panels e and f of Figure 3).

**Nile Blue A Staining for Lipid Localization on Kernel Surfaces.** The identification and localization of lipids following staining with Nile Blue A on whole kernels is challenging. The kernels tend to swell in the solvent, water, but water is required for the solubility of Nile Blue A. The fluorescence, however, is actually due to Nile Red, which is a component in Nile Blue A.[22−25] Nile Blue A is much easier to use, however, because it is soluble in water, does not inhibit the function of Nile Red, does not require the use of organic solvents, and does not require storage of its working solution at low temperatures in the dark. Nile Red selectively partitions into the neutral lipids and fluoresces yellow to red depending upon the relative hydrophobicity of the tissue environment. Despite its ease of employment, there are other challenges with Nile Blue A staining that include fluorescence quenching and competing autofluorescence of endosperm components, such as cell walls and proteins. Therefore, other lipid stains were investigated, and Sudan Black B was chosen for its relatively simple implementation.

**Sudan Black B for Lipid Localization on Kernel Surfaces.** Where the pericarp on whole kernels was intact, no staining with Sudan Black B was evident because the dye could not penetrate the thick, waxy cuticle of the pericarp. However, where the pericarp had been compromised, the stain penetrated and the dark blue color appeared on the kernels (panels a−c of Figure 4). Surface lipid was stained a deep blue by Sudan Black B in a single milled kernel, while the white,

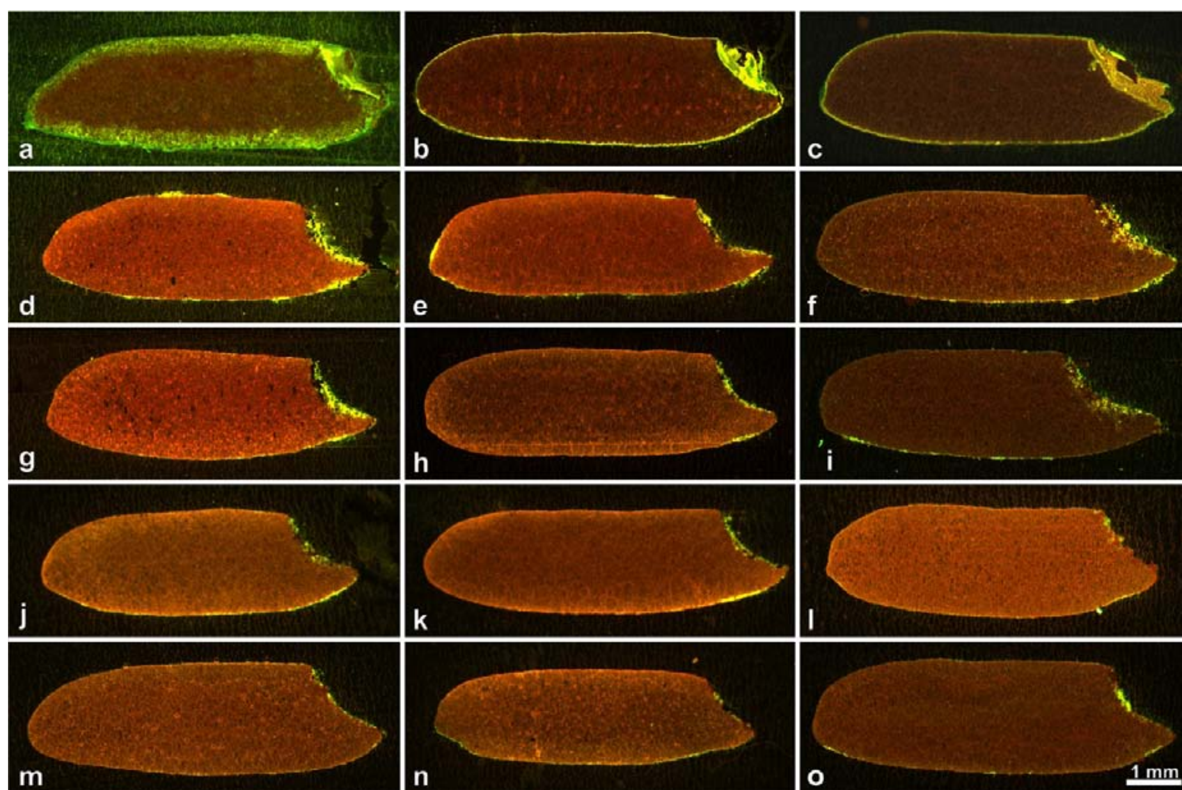


**Figure 5.** Longitudinal mid-kernel sections of whole grain and milled rice kernels stained with Nile Blue A for lipid (yellow material is neutral lipid). The sections were photographed using a fluorescence microscope using a combination filter with an excitation of 450−490 nm and a barrier long pass of 515 nm. The whole grains (a−c) show the location of lipid in the whole, unmilled kernel. The milled kernels (d−o) show the location of the residual lipid after milling. Brown rice kernels (a−c). Rice kernels milled for 10 s (d−f), 20 s (g−i), 30 s (j−l), and 40 s (m−o). The rice cultivars are Cypress (a, d, g, j, and m), Wells (b, e, h, k, and n), and an experimental hybrid (c, f, i, l, and o). The growing locations are Corning, AR (a, b, d, e, and g), Newport, AR (h, k, and n), and Stuttgart, AR (c, f, i, j, l, m, and o).

unstained endosperm provided good contrast for the dark stain (panels d−o of Figure 4). Sudan Black B was also superior for staining surface lipids because the solvent, 70% ethanol, did not induce swelling of the kernels nor did it solubilize lipids. Differences in the milled samples were obvious: samples milled for 10 s (panels d−f of Figure 4) stained the most, and those milled for 40 s stained the least (panels m−o of Figure 4), which clearly demonstrated differences in DOM. The samples milled for 20 and 30 s had intermediate staining relative to samples milled for 10 or 40 s (panels g−l of Figure 4). Cultivar differences were also demonstrated using Sudan Black B. Initially, Cypress milled more slowly than the other cultivars, followed by the experimental hybrid, where some pericarp remained after 10 s of milling, evidenced by the unstained area in Figure 4f. However, after 40 s of milling, Cypress appeared to have milled better than the other cultivars, as indicated by less staining from Sudan Black B (Figure 4m). This result, however, could be due to the limited sample size. The linear staining pattern was due to the lipid remaining in kernel depressions that ran along the long axis of the kernel. The protuberances were typically milled away, while material in the lowest depressions was left behind, following even the longest milling durations.

**Nile Blue A Staining for Lipid Localization on Kernel Sections.** Although Nile Blue A presented many challenges for the demarcation of lipids on the surfaces of whole milled kernels, it worked very well on sections (panels a−o of Figure 5). Sections contain much less tissue than whole kernels because they are only 10 $\mu$m in thickness, whereas a whole kernel is roughly 2 mm in thickness. Fluorescence intensity is partly related to the amount of material in a sample; therefore, there is much less fluorescence associated with a 10 $\mu$m section compared to that in a whole kernel. The pericarp surrounded the outer edge of the sections as a thin strip (panels a−c of Figure 5). Therefore, even with intact pericarp, there was much less volume associated with sections than with whole, intact kernels. Thus, because there was less residual pericarp to autofluoresce and the pericarp and lipid-containing aleurone layer were clearly distinguished in sections, there was less interference with the Nile Blue A staining. The longitudinal sections confirmed whole kernel-staining results, showing that lipid remained on the surface in various locations (panels d−o of Figure 5). The sections also demonstrated that the embryo was not completely removed and that variable amounts remained with the milled kernel (panels d−o of Figure 5). In addition, sections demonstrated that there was little or no lipid in the interior of the kernel, suggesting that total lipid content may also be used as a measure of DOM. In whole kernel preparations, because of the positioning of the kernel, it was not always possible to determine whether the embryo remained after milling. With sections, however, it was clearly evident that a large portion of the embryo remained and persisted throughout the duration of milling (panels d−o of Figure 5).

In conclusion, our results demonstrate that progressively decreasing amounts of lipid remain on the surface of the rice kernel after successive milling. This result confirms that the measurement of surface lipid content is a valid measure of DOM.[26] Virtually no lipid was found in the interior of the

kernel. Thus, total lipid content may also be used as a measure of DOM.[27] Lipid (and therefore, bran) remained in the linear depressions on the surface of the kernel after milling, indicating that the lipid does not solubilize and redistribute over the kernel as a result of milling treatment. Sudan Black B combined with low-magnification microscopy was a valid technique for observation of the surface lipids remaining on surfaces of whole kernels after milling. However, lack of staining did not necessarily indicate the lack of bran particles, because the pericarp did not stain and masked underlying lipids. Staining of sections with Nile Blue A was an effective technique that showed the depth of bran on sections of milled or brown rice kernels. These results provide proof of concept that residual lipid is a robust measure of DOM in rice.

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### Notes

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may be suitable.

The authors declare no competing financial interest.

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