

Project Title: Non-destructive detection of sun stress compromised apples (AP-19-104)

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Project Duration: 3-Year
Total Project Request for Year 1 Funding: \$88,947
Total Project Request for Year 2 Funding: \$91,545
Total Project Request for Year 3 Funding: \$94,246

Other related/associated funding sources: Awarded
Agency Name: USDA-ARS, In-house project
Amt. awarded/requested: \$61,313/3 yrs.
Notes: In-house project with complimentary objectives. Funds for storage maintenance and costs (\$8000/yr), supplies and materials (\$3000/yr), travel (\$5000/yr), and 0.1 FTE (co-PI).

Budget 1

Primary PI: David Rudell
Organization Name: USDA-ARS
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Item	2019	2020	2021
Salaries (WSU post-doc)	47,500	49,400	51,376
Benefits (WSU)	17,447	18,145	18,870
Wages (ARS part time)	10,000	10,000	10,000
Benefits			
Equipment			
Supplies			
Travel (for Lorenzo León)	2500	2500	2500
Miscellaneous*	11,500	11,500	11,500
Plot Fees			
Total	88,947	91,545	94,246

Footnotes: One-third instrument service contract (TFRL, USDA-ARS)

Objectives:

1. Determine best non-destructive methods to segregate sun stress compromised fruit.
2. Validate accuracy of non-destructive methods for detecting chemistries associated with solar stress.
3. Test if non-destructive sorting improves storage outcome for different sun stress related disorders.

Goals and Activities to be finished in this storage season:

In Year 3, a pilot study uses this index and classification protocol as a means of sorting Granny Smith apples from bins picked from 4 orchards and, then, assess external and internal quality following storage. We are using comparisons of orchards to refine and validate our sorting protocols and classification criteria. We are continuing to dissect tissue to confirm sun exposure assessment and sunscald prediction using hyperspectral imaging matches expected levels of peel chemicals associated with spectral changes and cumulative sun exposure. We will focus on those images that indicate the presence of yet unknown differences of peel chemistry.

SIGNIFICANT FINDINGS:

1. Indexes were determined that can be used to sort 'Granny Smith' fruit not already damaged by sun according to cumulative sun exposure using UV-Vis hyperspectral imaging.
2. Peel area can be classified according to cumulative sun exposure, sunburn presence, and sun scald risk.
3. A protocol for calibrating the index among orchards and seasons was developed and is under evaluation.
4. An image summarization protocol was developed to allow for a more usable datafile size.
5. Apples can be sorted at harvest according to sunscald risk, sunburn, and cumulative sun exposure and potentially other purposes.

METHODS*Hyperspectral imaging*

Apples are scanned using a Nano-Hyperspec VNIR imager (400-1000 nm), tungsten light source, and scanning bed (Headwall Photonics, Bolton, MA) for all experiments. Composite hyperspectral images (data cubes) have been used for Vis-NIR predictive model development based on multiple spectra. We have tested multiple models/indexes we developed as a means to sort fruit according to cumulative sun exposure. The general protocols developed are included in the following.

Objective 1: Determine best non-destructive methods to segregate sun stress compromised fruit.

In years 1 and 2 of the project, we imaged fruit from 3 different sun sensitive cultivars at harvest and then stored them in air for up to 5 months and assessing fruit finish and appearance defects monthly. Ongoing analysis of these and other hyperspectral images and spectra has been used to develop multiple indexes that could be used to sort sunburn, predict risk for sunscald and other sun-related disorders, and sort apples according to relative sun exposure. From this, we chose a simple index (chlorophyll a/carotenoids) that worked for assessing peel for sunburn presence, sunscald risk, and relative sun exposure of peel. We developed and are refining a protocol for categorizing fruit based on sun exposure index over the peel area of individual fruit (see in results and discussion) and are improving the process using multiple bins harvested in year 3 (see harvest details under Objective 3).

To improve our assessment of tree position (beyond disorder risk assessment) using this index, in Year 3, we harvested a set of fruit from large open vased trees while cataloging each apple's exact tree position from the most to least sun exposure. These apples were imaged and a subset of peel

sampled for comparison of actual content of natural chemicals (chlorophylls and carotenoids) used by the index with those estimated using imaging. We expect this will aid us in improving our index to allow for more categories of sun exposure (possibly more sorting line drops). This will also help us validate associations between estimated light exposure, our index, and tree position for continued development of this predictive sorting technique.

Finally, also in Year 3, 'Fuji' apples were harvested from whole canopies at Sunrise experimental orchard, numbered, imaged, and placed in air storage at 33°F for 5 months. We will analyze these in addition to Honeycrisp apples harvested and imaged in the previous years to adapt the cumulative light exposure sorting protocol for use on these varieties, albeit for potentially different disorders and purposes.

Objective 2: Validate accuracy of non-destructive methods for detecting chemistries associated with solar stress.

In Year 2, peel samples were collected from Granny Smith apples selected for different levels of sunburn that had been imaged at harvest. The purpose of these activities were initial steps in validating links among sun exposure, UV-Vis hyperspectral images, and target natural peel chemicals linked with sun exposure reported in our earlier results (AP-16-102).

In Year 3, we are refining our analysis by using hyperspectral imaging to dissect peel from Granny Smith apples before symptoms appear. By doing this, we may be able to more exactly, compared with only contrasting sun facing and shaded sides of the apple, find cuticular changes associated with sunscald development and clues that lead to novel protective approaches that interrupt sunscald development. Another activity for Year 3 is analyzing peel from Granny Smith harvested from documented tree positions (see activity under Objective 1 for description). We will estimate levels the natural chemical targets used for our index—chlorophylls and carotenoids.

Objective 3: Test if non-destructive sorting improves storage outcome for different sun stress related disorders.

In year 3, 1 bin from each of 4 orchards was picked at commercial harvest. Orchards (Sunrise, Mattawa, Brewster, and Royal City) were chosen for different tree size, training system, and, consequently, relative sun exposure. Entire trees were picked into bins to get a full representation of sun exposure across each training system. Apples were marked on 1 side, individually numbered, scanned using the UV-Vis hyperspectral imager, boxed, and placed in air storage at 33°F. Fruit have been categorized using our protocol and will be compared with outcomes at 5 months. Appearance (including disorders) and quality (firmness, soluble solids, and titratable acidity) will be evaluated. Our main purposes are to validate that we accurately separate out sunscalded peel using our protocol and determine if fruit quality is more consistent among fruit in the same sorting category.

RESULTS AND DISCUSSION

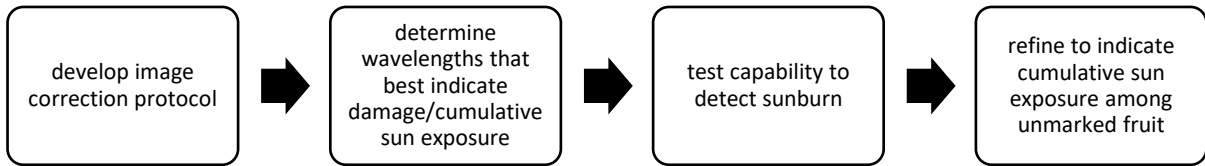


Figure 1. Initial steps used in developing a protocol for sorting apples at harvest according to cumulative sun exposure.

Image correction protocol and spectral regions indicating cumulative sun exposure (Figure 1)

A hyperspectral camera uses spectra from every pixel within the image. As not all information within the image is useful, adjustments must be made to ensure only good information is incorporated in the prediction (not shown). This was accomplished by manual annotation using the software SuperAnnotate (Sunnyvale, CA) to remove all glare pixels and those that were too dark. The remaining information was used to construct a model (first) that detects sunburn and, consequently, sunscald. This was completed using a population of Granny Smith apples containing severely, moderately, and lightly sunburned fruit as well as exposed, yet healthy, fruit. Unusable regions were removed and models generated to best detect sunburn and predict sunscald using a process called Convolutional Neural Networks (CNN) analysis. Four models were generated that indicated sunburn

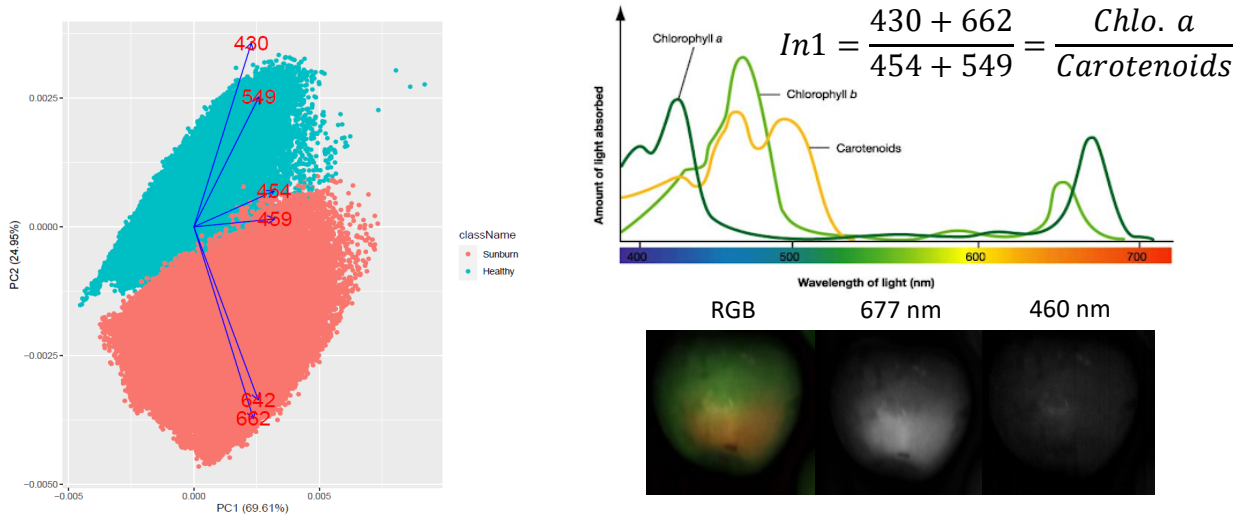


Figure 2. Determination of spectral regions most indicative of tree position. A principal component loading plot indicating spectral bands associated with chlorophyll and carotenoids are most responsible for indicating sunburn compared to unmarked fruit (left). Differences of absorbance in these spectral bands are illustrated in the upper right graph. To demonstrate in an image, in the 430-500 nm band, the increased levels of carotenoids in sunburned tissue counter the diminished chlorophyll levels leaving us with absorbance in both areas and a solid dark picture (bottom right). However, at 642 and 662 nm, chlorophyll content can be estimated without interference from the carotenoids (top right). Consequently, the ratio between reflectance at 642 and/or 662 nm and 549 nm (chlorophyll/carotenoid) provides a good estimation of sun exposure in Granny Smith.

in the images based on differences in 2 main regions in the visible-near infrared spectrum (for ‘Granny Smith’) using 91 equidistant wavelengths in the range from 600 to 800 nm. Based on 2 of the models, apples could be sorted for, for instance, sunburn with very low false positive (0.87-1.6%) and false negative (0.29-0.49%) rates.

While this may be useful and would remove all sunburned apples, including those with nearly unnoticeable levels, as well as those most likely to sunscald, our goal was to determine cumulative sun exposure, even among the “clean” fruit. We chose to base this analysis on differences in levels of metabolites that we know are associated with tree position (Musacchi et al, PR14-108A) and sun exposure (AP-16-102, Racsko and Schrader, 2012; Grandón et al., 2019). This was validated by an evaluation of readable regions from the sunburn gradient population mentioned above. In this way, we are using a non-destructive imaging system to analyze levels of chemicals that we destructively analyzed in prior work. Spectral bands resulting from peel chemicals that absorb light at 430 nm (chlorophyll a–green pigment), 459 nm (chlorophyll b–green pigment), 454,549 nm (carotenoids–orange and yellow pigments), and 642 nm/662 nm (chl a/chl b–upper bands, green pigments) provided a good separation of sunburn and healthy peel (Figure 2). This indicates we can expect carotenoid (orange color) levels to be high and chlorophyll (green color) levels to be relatively lower as peel is progressively more sunburned while healthy tissue has a higher ratio of either or both of the chlorophylls to carotenoids (Figure 2). Consequently, the chlorophyll to carotenoid ratio may be expected to be lower, even in entirely unmarked peel, with greater cumulative sun exposure. To test this, Granny Smith apples from the progressively sunburned population were compared with the front and back side of apples from a commercially picked bin that were then labeled and imaged monthly (front and back) as well as photographed for appearance rating. Usable pixels from each of the populations were analyzed according to three different indexes to create logistic models testing the accuracy of the characterization and, then, actually characterizing each pixel. From this analysis, we could determine the range of reflectance values and the relative area of peel represented in each range with index providing the greatest range of values and, possibly, the most capacity to categorize fruit according to cumulative sun exposure. Comparison of the index curves from all 3 populations highlights this factor with sunburned population having the highest range, and the exposed and unexposed sides of the random population from the bin having relatively intermediate and narrow ranges of values, respectively. The diminishing ranges of index values within these populations with sun exposure indicate the utility of characterizing a random population, such as the apples from the bin, according to relative sun exposure using this index. A “cluster” analysis of only exposed side images confirms the former observation (see Year 2 continuing report). Profiles of the index value Granny Smith peel from the exposed side and shaded sides are different (Figure 3). Apples that would later develop sunscald have a much broader range of index values indicating diverse light exposure that would be expected if sun damage (sunburn) was present at harvest. Furthermore, profiles of the index value from total peel area (peel represented in whole bin) of 4 orchards harvested in Year 3 indicate that orchards with larger variability of shading have a greater index range.

Finding sun stressed peel using sun exposure index

Further image processing as well as development of a fruit categorization logic and strategy was required before this index could actually be employed to sort fruit according to sun exposure. The sorting criteria should also account for useful outcomes such as removing sunburn and isolating sellable fruit that has a higher risk of developing sun stress related disorders such as sunscald. To develop the categorization protocol, we used the same progressively sunburned Granny Smith population coupled with whole bins of fruit in 2019 and 2020. Fruit from bins were numbered for tracking throughout the cold chain. Fruit were all imaged on both sides at harvest.

Approaches to establishing useful index thresholds (those that are indicative of sun exposure and/or sunburn presence or sunscald risk) used modeling (see sunburn determination protocol above) or index thresholds based on observation. Predictive modeling of sunscald risk establishes index thresholds based on prior years' comparisons of index values with actual sunscald incidence. The strengths of this method include accuracy and robustness across the whole year for prediction within the population used to construct the model. A major weakness of this approach is that, for the model to remain predictive, other variables such as image characteristics and relative year-to-year or even orchard-to-orchard chlorophyll and carotenoid levels must be similar. Given that it can be easy to see the color differences of Granny Smith among orchards, robust prediction among lots is not likely using this approach.

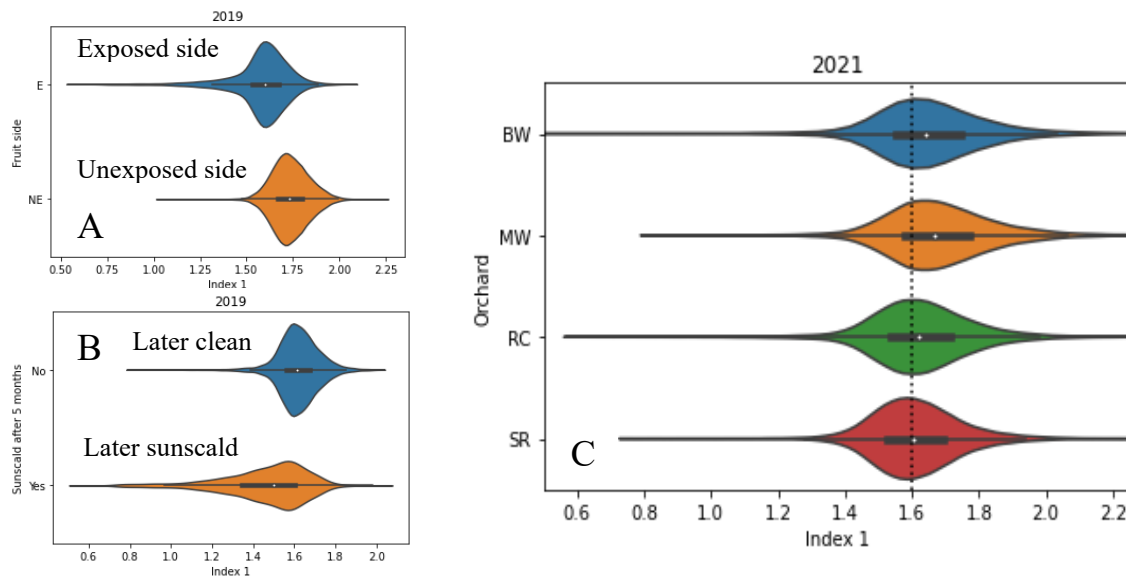


Figure 3. Aspect (side) with respect to sun (A), sunscald risk (B), and tree training (C) impact index values over the entire population. Light exposure alters index as indicated when contrasting sides apples. Variability of sun exposure such as found in fruit with higher sun exposure that leads to sunscald as well as canopies with more shading have a wider range of cumulative light exposure and, consequently, a wider range of index values. Density plots indicating relative differences of an index that estimates over 1 bin of Granny Smith apples per orchard in 2021. Whole trees were harvested to include the impact of light exposure throughout canopies from orchards with different training and management strategies. Orchards included were large free-standing open vase canopies from Brewster (BW–blue), medium canopies with shading from Mattawa (MW–orange), vigorous canopies on V trellis from Royal City (RC–green), and a fruiting wall from the WSU Sunrise (SR–red) experimental orchard. The widest point on each plot indicates the index value of the most peel area and the range of values estimates the variability of light interception in each population. For instance, the widest ranges of values are exhibited by the populations from Brewster and Mattawa which had the largest trees and the largest variability of light exposure. Diverse populations will also help us continue to test the risk assessment model and the impacts tree position may have on cold chain performance.

Basing index thresholds on “rules” set by biological observation could overcome these issues and, if based on simple observations, could provide a means to calibrate the index for each new population. To accomplish this, we set index thresholds according values of pixels (the smallest unit of image area) within moderate sunburn (assumed to be rejected due to subburn), mild sunburn (mild yellowing but not rejectable “sunburn”), and a the threshold for direct sun exposure (exposed vs shaded side). Risk assessment for sun-stress related injury, sunscald in this case, is based on years of evidence indicating sunscald will develop in regions of mild or worse sunburn with the mild sunburn remaining sellable, yet not storable (Grandón et al., 2019; AP-16-102). The basis for this risk assessment can be best visualized by pseudocoloring using index intensity (least intense color=lower index value=greatest sun exposure) at harvest compared with the sunscald (and superficial scald outcome) (Fig. 4). If this calibration works, index values will be anchored to key observable points across the index range and most variation among years and orchards/lots/blocks will be corrected so as to not compromise the assessment. To test this, for the 2021 harvest we deliberately chose 4 orchards with very different training system. This test is ongoing.

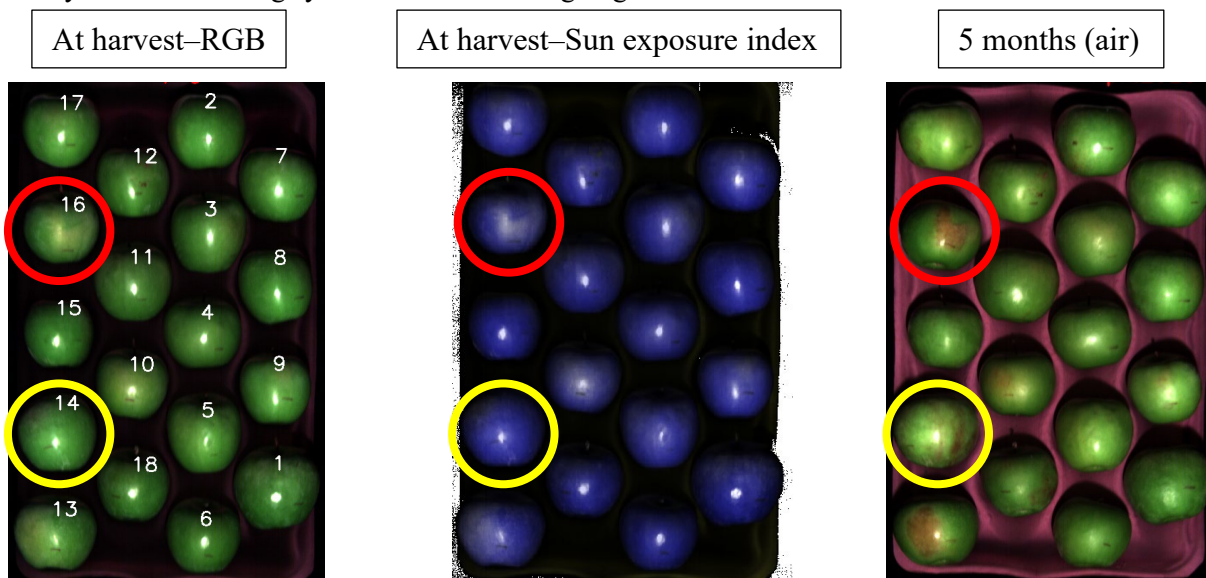


Figure 4. Pseudo-colored at-harvest image indicates where sunscald, but not superficial scald, will develop. Granny Smith apples real color at harvest (left), pseudo-colored image at harvest using cumulative sun exposure index where lighter blue color indicates a lower index value and elevated sun exposure and disorder outcome (both sunscald and superficial scald) after 5 months storage in air (right). Regions of lighter blue color at harvest indicated regions of sunscald (example indicated in red circle) after storage. Other examples are evident in the image. As expected, superficial scald, which is typically eliminated in peel with high sun exposure, was not present in regions of lighter blue color (example indicated in yellow circle).

A protocol for non-destructive risk assessment of sunscald on a fruit-by-fruit basis

Calibrating thresholds according to these criteria only works to evaluate cumulative sun exposure or disorder risk in a discrete area. In reality, it takes thousands of these points or pixels to create an image of a single apple and the ratio of pixels within threshold ranges over the entire fruit as well as the pattern can also be informative. Furthermore, a detailed image is unwieldy and would make transformation of any image into something useful for rapid sorting difficult.

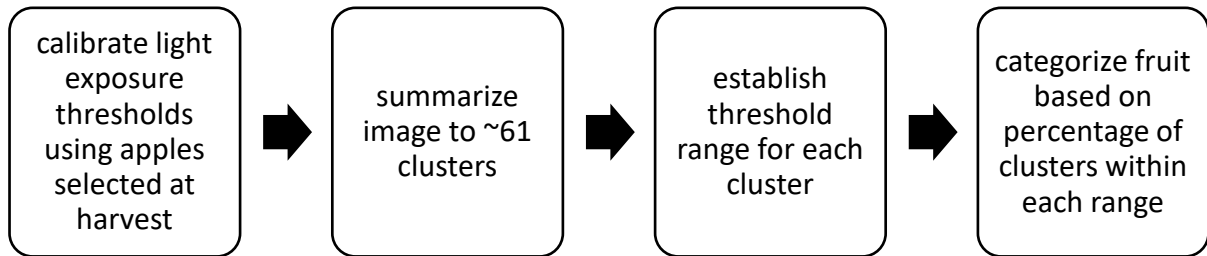


Figure 5. General process for categorizing fruit according to sunburn presence, sunscald risk, and relative sun exposure. Thresholds are calibrated for each orchard and this calibration is used to determine regions of Granny Smith peel that are impacted differentially by sun exposure. Based on this, apples can be categorized for sun-related disorder risk or even, potentially, relative tree position.

To begin to resolve these issues, we are defining a process that summarizes the image into a more manageable datafile size, easily calibrates the risk assessment and relative cumulative light exposure thresholds, and logically determines which of 4 categories the fruit fits into with respect to disorder presence or risk (Fig. 5). First, total pixels are automatically summarized into approximately 61 pixel “clusters” per fruit, depending upon fruit size. This affords a manageable datasize without losing the required spatial information for sun exposure assessment. The pixels are then coded into colors based on thresholds established for this population (black=sunburned/reject; red=high risk; yellow=exposed/low risk; green=less exposed/very low risk) (Fig. 6). A tool was developed to visually perform calibration using test apples selected for moderate sunburn, mild sunburn, or clean apples for shade/sun side comparison. We envision this could be performed onsite from quality control samples selected for any population. Next, criteria were developed based on pixel color fit individual apples into categories. The “reject” category (category 1) contains apples that have any sunburn that would be considered a defect. Any area of sunburn was considered a defect. However, based on our assessment of “noise” within the entire sorting system (the minimum clusters per color that may be in error). The “high risk” category (category 2) is also defined by a minimum amount of red clusters as any area of sunscald will be considered a defect if it develops later in the cold chain. Categories 3 (“exposed–low risk”) and 4 (“unexposed–very low risk”) more or less (respectively) than 65% green clusters. This is only a general outline of our strategies and we expect these variables to change as we incorporate our 2021 data and refine our process.

Categorizing a training set containing different sunburned apples with respect to severity as well as clean apples (to help develop the sorting process) from 2019. Our sorting protocol indicated 2, 55, 18, and 26 % of fruit were in the very low, low, high, and reject categories with 92 % of sunscald was found in the high and reject categories (Table 1). This is in line with what we could expect with a highly sun exposed population. We are continuing to improve the classification using subsequent years' data where we have followed a bin of Granny Smith in 2020 and are following 4 in 2021. We

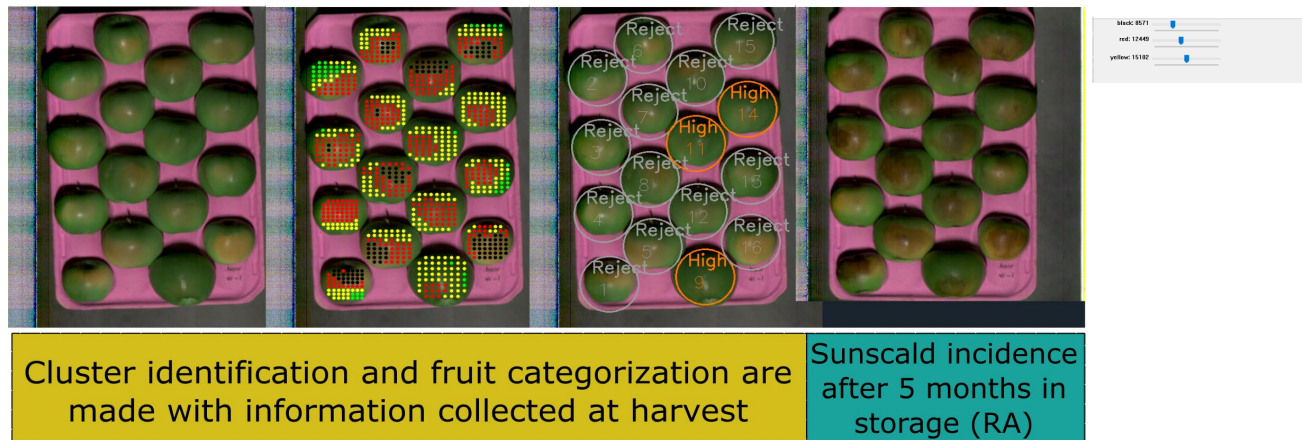


Figure 6. Example of classification protocol using moderately (defective) sunburned Granny Smith apples to demonstrate color range coding for estimating sunscald risk and sun exposure. Green colored clusters represent peel with the least sun exposure while yellow indicates peel estimated to have more sun exposure. Red clusters indicate high risk for sun scald and black clusters indicate regions that should immediately be rejected for sunburn. The presence and or percentages of different colored clusters on the fruit surface is the basis for categorizing the fruit. The tool on the right side indicates a protocol for calibrating the range for each risk/sun exposure range in each orchard using fruit such as these.

expect to refine this process to incorporate all of the sunscald in the high risk category while keeping our logic firmly linked with the observed biology and horticulture so that it remains robust across multiple orchards and years. To confirm links with actual measurements of the carotenoids and chlorophyll, peel samples from a subset from 2021 have been sampled and are being analyzed alongside the images. Such verification is necessary to directly confirm links between the destructive and non-destructive estimations. Furthermore, patterns we can see in the peel using the imager suggest that we can detect the boundaries of future sunscald symptoms. We intend to use imaging technology to detect further differences linked with cuticle changes associated with sunscald symptom development. In 2021, we are also looking into similar classifications using data we have collected on Fuji and Honeycrisp. We are determining whether this classification system can be used estimate risk of appearance defects and disorders associated with these cultivars.

Table 1. Categorizing Granny Smith apples according to sunscald risk and sunburn leads to improved consistency with regard to sunscald outcome in different sunscald risk categories. Apples were harvested from “fruiting wall” trained Granny Smith trees at the Sunrise experimental orchard although the population was intentionally enriched with fruit containing sun damage to better train our protocol. We expect to improve this using our dataset from bins of fruit from 4 orchards to be analyzed in 2022.

	<i>V.Low</i>	<i>Low</i>	<i>High</i>	<i>Reject</i>
No sunscald	13	391	90	30
Sunscald	0	17	41	162
Total fruit	13	408	131	192

Beyond sun-related disorder risk assessment

Other activities began during the 2020 season and wrapping up in the 2021 season include estimating differences in cold chain performance based on cumulative light exposure. Parameters include firmness, soluble solids and titratable acidity on half of the bin harvested in 2020 and all 4 bins harvested in 2021. This may have applications for apples but prior evidence indicates that a more useful application would be pre-sorting pears.

Potential for improving capacity and accuracy of sorting apples according to cumulative light exposure.

Success using the current index (above) exceeded our expectations so we chose to focus our efforts on making that sorting system work. However, the promise of even more accurate sorting measuring levels of light exposure related natural chemical targets in the near ultraviolet range is still worth pursuing even though its application is further from practice than imaging carotenoid and chlorophyll content using the visible wavelengths of the current index. Nevertheless, we made progress in showing that these chemical targets can be measured using a novel ultraviolet (UV) hyperspectral camera alongside a powerful UV light source to image in spectral regions where other chemicals linked with sun exposure absorb light as well as potentially identify new spectral bands that indicate risk for peel defects. Spectral regions within the higher wavelengths (400-500 nm), detected using this imager, differentiate sunburned from healthy peel. This difference is based on contrasting levels of carotenoids detected as also detected by the Vis-NIR sensor (the current index) and, potentially, other components visible outside the sunburned region (Figure 7A). Differences in our target region, below 400 nm, were not detectable when imaging whole fruit.

Other natural peel chemicals absorb UV light between 350 and 360 nm but have been challenging to image in whole fruit using the vis-NIR system. Consequently, we performed a series of tests to determine whether detection is possible by altering our light source or camera/light source configuration. In the first test, solutions containing pure target compounds were drawn onto white filter paper and imaged indicating most of the compounds that may be visualized in this spectral region were detectable and our principal targets, the flavonol glycosides (rutin), were not interfered with from other common metabolites (Figure 7B). Another test of different concentrations of rutin painted onto filter paper indicated that the imaging could indicate amount of this class of chemicals within a range typically found in apple peel (Figure 7C). Finally, a test of ethanolic extract from the sun exposed and shaded side of apple peel, painted onto filter paper, and then imaged indicated that

levels of flavonol glycosides were far lower on the unexposed than the exposed side. Given these results our earlier success on whole apples with weaker source light and a simpler camera, we expect interference in the target wavelengths may result from absorption of light by chemicals in cell layers below the peel, confounding differential absorption by target compounds in the peel.

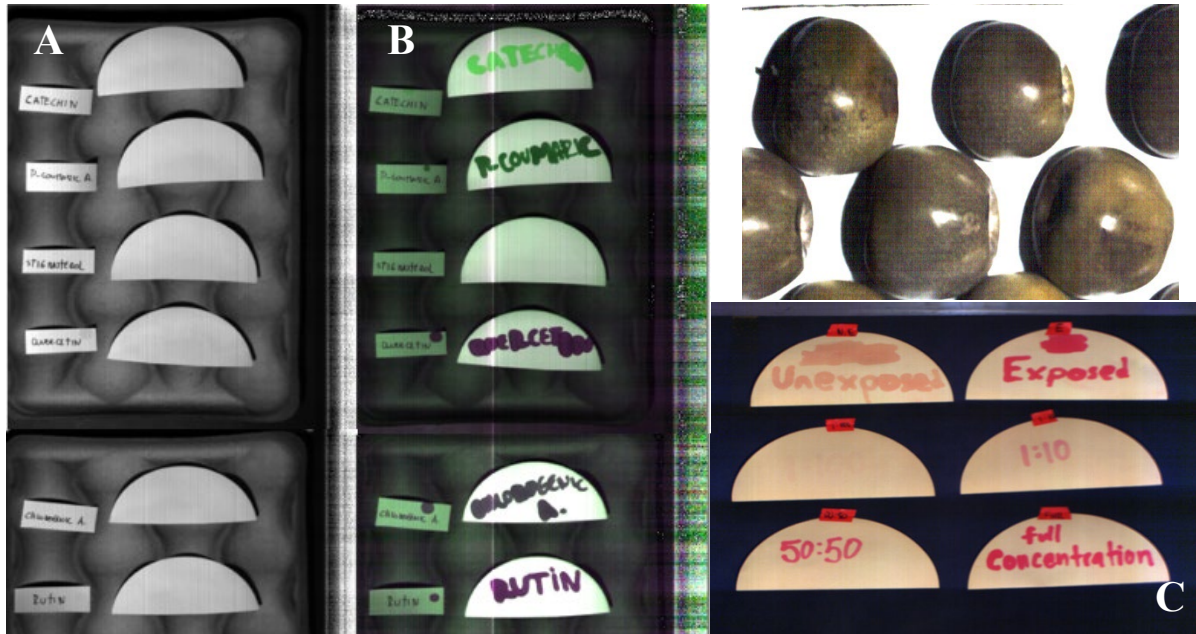


Figure 7. Ultraviolet-visible hyperspectral imaging may improve accuracy of non-destructive assessment of cumulative sun exposure. Images of apple between 400 and 500 nm using a UV-vis hyperspectral camera coupled with a high intensity UV light show pattern on the peel not visible to the naked eye (A). Pure natural chemicals painted onto paper cannot be seen with the naked eye but can be imaged at different wavelengths in the UV range (B). Rutin is a target chemical and spectra of other chemicals in that range do not interfere with imagining. The reflectance intensity of rutin solutions painted onto paper (“full concentration”, 50:50, 1:10, and 1:100) diminishes with dilution indicating the images are quantitative (C). Granny Smith peel extracted with ethanol from the “unexposed” and “exposed” sides and imaged at the wavelength that rutin and related compounds (flavonol glycosides) absorb most indicates more absorbance on the exposed side and, therefore, more of these compounds.

Project Title: Non-destructive detection of sun stress compromised apples (AP-19-104)

Executive Summary

Keywords: cold chain, sun stress, fruit finish, sorting, sun scald, risk assessment

Abstract: Apple peel appearance and chemical changes result from different relative sun exposure. This can lead to sun scald and other defects during the cold chain. Using sun exposure-related natural peel chemical targets reported in our earlier project, we have developed an index that can be used to establish relative cumulative sun exposure of peel using non-destructive imaging. With this index, we have developed and are refining a protocol for estimating whole-apple relative sun exposure that can be used to sort apples according to sunburn symptoms, high sun scald risk, and different relative sun exposure. Rather than determining a sorting protocol based on the capacity of a specific imaging technology, our index is rooted in biology, tying our collective findings linking natural peel chemicals with sun exposure, finding non-destructive methods to estimate levels of these chemicals, determining an index using these targets that best estimates relative sun exposure, and, finally, developing a sorting procedure based on index levels over the entire surface of fruit. Refinement of this protocol is ongoing at the time of this report.

Project outcomes:

1. Identification of an index using optical properties of natural peel chemicals that can be used for non-destructive sorting.
2. Associating index values with peel with sunburn, sunscald risk, and cumulative sun exposure.
3. A sorting system that estimates sunburn, sunscald risk, and, potentially, tree position.

Significant Findings:

1. Indexes were determined that can be used to sort ‘Granny Smith’ fruit not already damaged by sun according to cumulative sun exposure using UV-Vis hyperspectral imaging.
2. Peel area can be classified according to cumulative sun exposure, sunburn presence, and sun scald risk.
3. A protocol for calibrating the index among orchards and seasons was developed and is under evaluation.
4. An image summarization protocol was developed to allow for a more usable datafile size.
5. Apples can be sorted at harvest according to sunscald risk, sunburn, and cumulative sun exposure and potentially other purposes.

Future Directions:

1. Adapting sorting protocol for existing sorting lines.
2. Sorting at-harvest in packing facility or even field sorting to reduce transport and storage of already sunburned fruit as well as storage of fruit that is at a high risk of developing peel disorders.
3. Adapting sorting protocol to be used for other cultivars.
4. Optimization/validation of this protocol for sorting apples and pears according to tree position for potentially more consistent postharvest performance.