Development of a Colorimetric Test Kit to Determine Enzymatically Produced Pyruvic Acid in Sweet Onions

By

Dawn C. Merrill

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APPROVED:

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Dr. Kim Anderson, Primary advisor Date

_____

Dr. Michael Penner, Secondary advisor Date

_____

Dr. Kate Field, Director of Bioresource Research Date

I understand that my project will become part of the permanent collection of Oregon State University, Bioresource Research. My signature below authorizes the release of my project to any reader upon request.

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Dawn C. Merrill, Author Date

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By

Dawn C. Merrill *, Michael H. Penner †, Kim A. Anderson ‡

Bioresource Research, College of Agricultural Sciences

† Department of Food Science & Technology

‡ Department of Environmental and Molecular Toxicology

Oregon State University, Corvallis, OR 97331

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Abstract

Sweet onions are valued for their unique flavor and praised for their health and dietary character. Onion sweetness is perceived as the degree of pungency decreases. Pungency can be quantitatively determined in onions by measuring enzymatically produced pyruvic acid. The onion industry quantifies a sweet onion as one having a pyruvic acid concentration of 1 – 4 µmol/g. Identification of pyruvic acid is accomplished by reacting onion extract with 2, 4-dinitrophenylhydrazine (DNPH) to produce a colored adduct that is measured spectrophotometrically. Currently, onion processors outsource pungency analysis to a laboratory in Texas. The cost and turn around time are both high. Satisfying the need for a modified method, where samples can be analyzed quickly on site, is the goal of this research. The laboratory method produced data qualifying sweet onions. Averages of 15 samples resulted in 4.05 ± 0.48 with a percent recovery by over-spiked samples of 104% ± 10.4%. Check standards reveal a percent recovery of 98% ± 0.78%. This method is reproducible and adaptable for a quick, field test kit for onion breeders. The test kit identifies pyruvic acid with the use of the DNPH color indicator, and qualifies sweet onions by comparing samples to a provided color chart. The test kit proposed reduces the cost of sweet onion analysis significantly and decreases the overall turn around time for pungency analysis.

Key words:

Pyruvic acid; onions; pungency analysis

Introduction

Onions are a food valued for their distinctive flavor profile and overall health benefits. Onions (*Allium cepa*) are members of the Liliaceae family, a family which also includes chives, garlic, leeks, and shallots. Worldwide, onions are regarded as a staple in many cuisines, and are used in food dishes to enhance overall flavor.

World onion production is high. The United Nations Food and Agriculture Organization (UNFAO) reports that approximately 175 countries worldwide grow an estimated 6.7 million acres of onions total (*1*). The United States is ranked third of onion producers in the world after China and India, respectively. Domestically, the United States onion crops account for 7 percent of the world's total onion production, with the value exceeding $4 billion USD retail in 2008 (*2*). According to the National Onion Association (NOA), the average annual onion consumption calculates to approximately 13.7 pounds of onions per person across the world (*2*).

Onions are associated with human health benefits due to their nutritive and phytochemical properties. Organosulfur compounds such as thiosulfinates (formed from flavor precursors) have been linked to lower blood pressure, cholesterol, and lower risk of cardiovascular disease (*3*). These sulfur compounds possess anti-microbial activity, anti-inflammatory benefits, anti-allergic properties, and aid in digestion. In addition, studies suggest flavonoid compounds such as quercetin provide protection against certain cancers by suppressing tumor growth due to their antioxidant activity (*4*).

Varietals range from yellow, white, red, green, pearl, and boiler onions. Onions are categorized by freshness. A typical ‘storage’ onion generally contains less moisture, stronger flavors, and can be recognized by its paper-like layers surrounding the bulb. Fresh onions lack that paper-like skin, and generally have higher water content. Fresh onions are milder in flavor, and are often regarded as “sweet.”

Sweet onions are a varietal of onion with unusually low pungency when compared to typical onions. Onions are classified by their level of pungency. A high pungency onion is said to give a strong, sharp, acrid sensation, whereas a lower pungency onion would impart a sweet flavor. Sweetness in onions can vary by cultivar, environmental and growing conditions, size, storage time, and vary in different parts of the bulb; however almost 90 percent of these sweet onions are yellow varieties (*1*). The onion industry quantifies sweetness in terms of µmol/g fresh weight, and concludes 1-4 µmol/g pyruvic acid qualifies a very mild sweet onion (*5*). Similarly, an onion containing 5-7 µmol/g pyruvic acid is qualified as a mild sweet onion, an onion with 8-10 µmol/g is intermediate, and an onion with greater than 15 µmol/g is said to be a highly pungent onion (*5*). Also contributing to the sweetness in onions are the sugars glucose, fructose, and sucrose. It is the combination of pungency and sugar that classifies a sweet onion where ideally a sweet onion would have high levels of sugars and lower levels of pungency (*6*). A few examples of sweet onion include Walla Walla sweets, Georgia Vidalias, Texas 1015, and Maui sweets. After being first recognized in the late 1800s in Bermuda, Texas, sweet onions quickly became a popular

commodity. The growing consumer awareness surrounding sweet onions caused the onion industry in the United States to create breeding programs to mass produce these highly desirable onions. The onion industry adapted a testing program to analyze pungency in onions as an effort to certify sweet onions and market those at a higher cost. Currently, this method of pungency analysis is done through a third party, causing a considerable delay in processing. Analysis of pungency in onions is currently being conducted by the Texas AgriLife Research & Extension Cooperative for onion processors. This outsourced method is both expensive and time consuming. A member of the cooperative will receive a fee of $60 per one of sample analysis and receive results within one week. Specifically, onion samples received on Monday or Tuesday will be analyzed by Friday (*5*). Shipping increases the wait time for results, and raises the fee, which does not factor shipping costs to send samples to the facility in Texas. The need for a fast, accurate, and simple field test kit for the determination of sweetness in onions is apparent.

Sweetness and pungency are measured by flavor compounds existing in various concentrations in an onion. Flavor in onions is derived from sulfur compounds taken up from the soil during its growth period. Sulfate (SO_4^{2-}) is first reduced to sulfide (S^{2-}). Through the processes photosynthesis and total assimilation, the amino acid cysteine is formed. A tripeptide of cysteine, glutathione, becomes the parent compound of three specific flavor precursors: 1-propenyl cysteine sulfoxide (PrenCSO), methyl cysteine sulfoxide (MCSO), and propyl cysteine sulfoxide (PSCO) (*7*). All exist in decreasing concentrations,

respectively. These flavor precursors, collectively known as S-alk(en)yl cysteine sulfoxides (ACSOs) are stable in the cytoplasm of onion tissue cells. Concentrations of ACSOs vary based on the unequal distribution within the onion bulb. More specifically, higher concentrations of flavor precursors exist in the interior base of the bulb (*7*). Once an onion cell is disrupted, the enzyme Alliinase (which exists in the cell vacuole) reacts with the ACSOs. A hydrolysis reaction occurs and several products are made. Figure 1 demonstrates the enzymatic breakdown of the ACSOs into stoichiometric amounts of sulfoxides, ammonia, and pyruvic acid. These volatile sulfoxide compounds are further broken up to produce thiosulfinates, including a lachrymatory compound that produces a tearing effect in the eye. In the same reaction, pyruvic acid and ammonia are produced in equal molar amounts to the ACSOs. Although onion pungency is directly related to the amount of sulfoxide compounds produced in this reaction, due to their instability, they are undesirable to analyze. The same is true for ammonia, which exists as an endogenous compound. Ammonia is not an accurate representation of pungency, and therefore pyruvic acid is a model compound for analyzing pungency in onions. The amount of pyruvic acid generated enzymatically upon onion homogenization is a good measure of the action of alliinase on the flavor precursors and has been shown to be correlated with perceived onion pungency (*8*). Understanding and capitalizing on this specific chemical reaction is the basis of this research.

Quantitative laboratory analysis of pyruvic acid is generally done using spectrophotometry. Spectroscopy in the visible range, that is 350nm to 750nm, is

a commonly encountered technique in food analysis. In a typical assay, light is transmitted at a specific wavelength through a cuvette containing a colored sample. The amount of light absorbed depends on the concentration of the absorbing material in the sample. The greater absorbance value will correlate to a greater concentration of the compound being identified.

Standards are used to create a calibration curve. A graph is plotted to compare sample results to known concentrations based on the Beer-Lambert Law, also known as Beer's Law. This law of spectrophotometry implies that absorbance of a colored solution is directly proportional to the concentration of the analyte in the sample by the equation:

$$A = \varepsilon bC$$

where A = absorbance, ε = molar absorptivity of pyruvic acid at 515nm, b = pathlength (cm), and C = analyte concentration. The analyte in the present case is pyruvic acid.

The goal of this research was to develop an easy test kit for field use. Investigation of other possible methods for determining pungency include the determination of pyruvic acid by HPLC (*9*), ACSOs by Sulfur Analyzer (*10*), pH by potentiometry, and gas volatiles by gas chromatography (GC) (*11*). While the instrumentation used in these methods provided accurate quantification of pyruvic acid and were successful in streamlining pungency analysis; they do not align with our goal. In addition, the primary purpose of the test kit centered on the ability of an onion processing plant worker to replicate the method, thus HPLC, GC, etc. were not considered in this research. Sweetness in onions can also be

measured by total sugar content. This technique measures sucrose concentrations (°Brix) and could be used in conjunction with pungency analysis, but is not necessary. While the advantages of this technique are that it is rapid and accurate, relevant disadvantages exist. Refractive index will vary with respect to concentration of the compound of interest, temperature, and wavelength of light (*12*). A refractometer is necessary in this analysis, and therefore was not investigated as a potential method for the development of the test kit.

Two objectives govern this research project. They are to determine an appropriate laboratory colorimetric method for detecting pyruvic acid in onion tissue, and modify that method to develop a fast, field applicable test kit for analyzing sweetness/pungency in onions.

The first objective in this research modifies a known method created by Schwimmer and Weston (1961). They report that 2, 4-dinitrophenylhydrazine (DNPH) reacts with pyruvic acid to form a colored adduct which can be measured spectrophotometrically using a wavelength of 420 nm (*13*). Specificity is crucial when choosing a color reagent. In this case, DNPH is used based on its specificity to bind to a ketone or aldehyde group. Pyruvic acid is a ketone. A modification of this procedure was developed by Anthon and Barrett in 2003. They designed an analysis using a 515 nm wavelength, and altered the ratios of reagent for reproducibility. Before then, the Schwimmer & Weston method was a standard in all rapid onion analyses. With the Anthon & Barrett changes to the method, more reliable determinations of pyruvic acid and onion pungency are

possible. For the simplicity and fast analysis of spectrophotometry, the combined methods became the starting point for this research project.

The second objective in this research is the construction of a field-applicable test kit for onion growers to quickly analyze their crop for sweet onions. Pyruvic acid is the standard indicator for sweet onions (pungency) in the onion industry. The criteria for this test kit are outlined in table 1.

The test kit will provide all necessary components for pyruvic acid analysis, including reagents, and will contain a color chart displaying colors representative of the assay. A field worker or onion processor can cut, peel, and quarter an onion for analysis, blend the sample, extract the juice, and add it to a vial containing the necessary reagents. Once the reaction occurs, the color in the test tube can be compared to a color chart. This technology will benefit and improve the analytical system onion growers currently use to determine the sweetness in their onions.

Materials and Methods

Onions

Onions were purchased based on availability at local grocery stores in Corvallis, Oregon between July 2009 and November 2009. Varietals included sweet, white, yellow (California brown), and generic boiler onions. Each onion was numbered and named based on location of the store it was purchased and the varietal. Quality of the product was a factor in choosing the onions. Onions were not chosen if they had obvious damage, discoloration, and/or stress from

aging. Size of the onion bulb was less critical for selection, though uniformity was a criterion.

Chemical Reagents and Standards

Sodium pyruvate ($C_3H_3NaO_3$) 99%+, Acros Organics was purchased as a standard from Fisher Scientific. CAS: 113-24-6, Catalog number: AC13215-0250, No.: 132150250. Standards were made by dissolving 1.1 g $C_3H_3NaO_3$ in 100 mL 18 mΩ H_2O to create a 0.1 M stock solution. Serial dilutions of the stock solution were made to prepare working standards from 0 µmol/mL to 10 µmol/mL. 2, 4-dinitrophenylhydrazine, reagent grade, 97% was purchased from Sigma-Aldrich. CAS: 119-26-6, product number: D199303-25G. Dissolve 0.025 g of 2, 4-DNPH by heating and stirring in 1.0 N HCl to make the necessary 0.25 g/L reagent. 6.0 g sodium hydroxide (NaOH), dry solid (CAS: 1310-73-2) was dissolved in 100 mL 18 mΩ H_2O to make a 1.5 N NaOH reagent.

Instrumentation

The Shimadzu UV-1700 spectrophotometer was used.

Onion Sample Preparation

Onions are stored at room temperature upon receipt until processing. Long term storage is not recommended as the quality of the product declines and does not accurately define the parameters of field analysis. At the time of sample preparation, the onions are first cut at the ends. The outer layers of the storage onions (paper-like) are discarded. The onions are then cut longitudinally and crosswise into quarters. Two quarters are used for two separate analyses. One quarter (labeled onion half “A”) is weighed using a Mettler balance and the mass

is recorded. A second quarter (labeled onion half “B”) is weighed and immediately placed in a microwave (power equivalent of 1,450 watts) and heated for 1 minute. The remaining onion quarters are evaluated for intensity of pungency using a sensory scale (table 2) and collected in a separate Ziplock© bag. Once onion half “A” is weighed, it is placed into a Warring blender with an equal volume of distilled water to gram of onion (ex. a 50g sample would be blended with 50mL distilled water). The onion is blended at medium speed for 1 minute. The onion slurry is then allowed to sit for 20 minutes while the enzymatic reaction occurs. For accurate data, it was experimentally determined that the onion slurry should not be filtered until all pyruvic acid is formed enzymatically (approximately 20 minutes). The onion sample is then filtered through two layers of cheesecloth, and centrifuged (max rpm = 4500) for 10 minutes. In an effort to reduce glassware, Styrofoam cups were used to collect the raw juice as demonstrated by Yoo, Pike, et. al., 1995 (*14*). Onion halves labeled “B” should sit for the duration of the onion half “A” processing, and once cooled, processed in the same manner as the fresh quarter. It is not recommended to store samples due to variation of data. It is suggested that onion processing and analysis occur within the same day to mimic the parameters of the field test kit.

For sample analysis, 25 µL of onion filtrate is placed into clean glass test tubes (according to SOP 2110.04) with appropriate sample name. Add 1 mL 18 mΩ H_2O, 1 mL 0.25 g/L 2,4-DNPH in 1.0 N HCl, and place in a 37°C water bath for 10 minutes. Add 1 mL 1.5 N NaOH to test solutions and immediately analyze by spectrophotometry.

Spectrophotometric Analysis

The wavelength chosen for this assay is 515 nm based on experimentally determined calibration sensitivity. Figure 2 demonstrates a reproducible standard curve at 515 nm. A blank cell containing milliQ H_2O was used to zero the instrument prior to sample analysis.

Calculating Absorbance

Once a standard curve is constructed, absorbance is proportional to the concentration of standards based on Beer's Law. Concentrations (in µmol/ml) of samples are obtained using data from the calibration curve.

Results & Discussion

Objective 1:

Method Development:

Schwimmer & Weston and Anthon & Barrett provide an appropriate starting point for this research. One significant change to the original Schwimmer & Weston (1961) method was the alteration of the ratio of DNPH reagent. This was necessary to optimize the reaction of DNPH with pyruvic acid in the sample. Schwimmer and Weston report that 0.0125% 2, 4 – DNPH in 2 N HCl was adequate to react with pyruvic acid (*13*). Pyruvic acid was the limiting reagent, so for greater accuracy Anthon & Barrett (2003) increased the DNPH reagent to 0.025% (1:25 P.A. to DNPH). 25% greater DNPH in solution reacts stoichiometrically with all pyruvic acid and leaves a lower amount of background absorbing material. The wavelength was altered to consider the linearity of calibration curve. The calibration sensitivity, defined as change in absorbance

per unit change in [pyruvic acid] concentration (*12*) became relevant. Due to the conflicting wavelength within the Schwimmer and Weston (1961) and Anthon and Barrett (2003) methods, a wavelength experiment was conducted. Figure 3 provides evidence that the calibration sensitivity of the pyruvic acid-DNPH adduct is not ideal at the wavelength of 420 nm. Similarly, figure 4 demonstrates that a wavelength of 445 nm produces a more linear curve although it too is not ideal. In addition, these two graphs demonstrate that the absorbance of the colored solution is greater than 1.0, which does not satisfy Beer's Law. Ultimately, the wavelength of 515 nm as described by Anthon & Barrett provides the greater absorbance measurement to the pyruvic acid-DNPH based on the chosen reagent concentrations. A standard curve was constructed before each analysis. Results show a R^2 value of 0.99 after each assay (as shown in table 3) and absorbance at 515 nm compared to concentrations of the standards as seen in figure 2. Although Anthon & Barrett (2003) provide evidence that a reproducible assay is reasonable; several considerations were investigated throughout the method development process to ensure appropriate guidelines for the formation of the test kit.

An observational study was designed to replicate the Wall & Corgan (1992) study to determine whether sensorial analysis can be used to determine pungency/sweetness. This was not a true sensory study as it did not include a trained sensory panel, rather it was introduced after the method to verify that sensory and concentration relate. This was to show that pungency analysis is best performed using human detection; however it is by no means quantitative,

as results show in figure 5. Results confirm pungency evaluation by smell alone may not accurately reflect the pungency that would be perceived by tasting (*8*). Limitations were based on one panelist responding to one stimulus. A numbered scale (table 2) was created to measure the aroma intensity of each onion bulb after it was cut. After the assay was performed, a graph was constructed to compare the concentration of the sample to the intensity score of each sample. While the human nose is used throughout the food industry as a preceptor of ripeness, quality, and can adequately detect flavor volatiles; it has not been shown to be as quantitative as analytical instrumentation. The study by Wall & Corgan (1992) did reveal a high correlation between flavor perception and pyruvate analysis; however the data above from this method were not as definitive. Results shown in figure 5 do not accurately predict concentrations of each onion sample. It is likely that a properly trained panel with appropriate blind study considerations would improve the correlations. The process of rating onions was not included in the final procedure.

Another consideration for method development was formed after results of preliminary data showed variation between onion replicates. In preliminary experiments, the onion homogenate was allowed to sit for one minute prior to filtration. A separate experiment was designed to test the hypothesis that the pyruvic acid was not reaching full formation after homogenization. Upon maceration, each sample was allowed to sit prior to filtration for a predetermined amount of time (in minutes). Time points chosen were 5, 10, 12, 15, 17, 20, 25, 30, 35, 40, and 45 minutes. An assumption was made that filtration through

cheese cloth stopped the enzymatic reaction. The experiment was designed as a robustness study, where the full formation of pyruvic acid would be determined. The expectation was a bell shaped curve where the formation and degradation of pyruvic acid (in time) would be evident. Figure 6 shows the concentration of enzymatically produced pyruvic acid versus these reaction times. Results from this experiment do not show clear formation and degradation of pyruvic acid, rather demonstrate that further studies are necessary. The filtering step using cheese cloth did not adequately stop the reaction so the results of this figure indicate total pyruvic acid concentration of pyruvic acid in one yellow onion. It is important to point out the total concentration of pyruvic acid only varies between 1 µmol/g throughout the entire experiment. This is not a large difference or variation, but it is notable as the difference in 1 µmol/g can be observed in the color spectrum. An average and standard deviation of the values is performed and compared to the data set for yellow onions and results are 5.82 ± 0.25 µmol/g of total pyruvic acid. This value compares to the data provided by the triplicate analysis, which are 5.85 ± 0.06 µmol/g (see table 4). Additional modifications such as faster filtration following the homogenization step, and eliminating the slow cheese cloth and centrifugation steps would likely reduce variation noted by this experiment. Additional analysis should test the likelihood that the necessary filtration can be accomplished using a 0.45 µm filter. This method will work based on the required final volume of sample is 25 µL.

Other considerations for variability include interfering material, onion cultivar, and storage time. Interfering material may vary in spectrophotometric

results between different onion samples (*15*). This can be defined as turbidity within the sample, bubbles formed inside the cuvette of sample solution, or other reacting ketone or aldehyde groups specific to this reaction. Also, the onions analyzed in this project came from local grocery stores, where the specific origin, cultivar, and growing conditions are not known. Storage of onion juice over extended periods of time showed considerable variability. Considering the parameters of the field test kit, it should be noted that onions should be processed and samples analyzed within the same day. Samples should be analyzed immediately after the addition of NaOH in this procedure. It has been reported by Anthon and Barrett (2003) that a noticeable turbidity formed when samples were allowed to sit for 1 hour or more after the addition of NaOH (*15*), so reasonable timing is critical. Also, the color is not stable and will photo degrade over days. For the purpose of future analyses, a standard operating procedure (SOP) was developed for the laboratory method for the determination of pyruvic acid in onion tissue (appendix A).

Sample Analysis

Each assay revealed the following data provided by tables 4 – 8. Table 4 presents the results of three replications using the same samples and demonstrates the mean concentrations of pyruvic acid (in µmol/g). A representation of a population of onions is observed after averaging the results of all three assays (n = 15) presented in table 5. The sweet onions have low pyruvic acid content, and fall in the range of very mild pungency as provided by the Texas AgriLife Research and Extension Center criteria (*16*). It can also be

concluded, based on the results of each analysis, that the sweet onion varietal purchased at a local retail store does not represent a broad range of pungency, and all classify as sweet. Check standard analysis was performed and resulted in a 98.0% ± 0.78% (table 9).

Endogenous pyruvic acid was also measured in conjunction to the total pyruvic acid. This background amount of pyruvic acid is subtracted from the total pyruvic acid to reveal the enzymatically produced pyruvic acid. A certain amount of pyruvic acid exists endogenously in onions as pyruvic acid is a major product formed via glycolysis and other metabolic processes. The heat provided in the microwave step denatures the enzyme, Alliinase, and therefore stops the reaction. Ideally, no enzymatically produced pyruvic acid is measured. Results from table 7 reveal higher background concentrations to that of the literature. It is likely that the amount of time taken from the maceration step to the microwave step did allow for some enzymatically produced pyruvic acid. Overall, the data provides the appropriate background information to further develop a test kit for onion breeders.

Objective 2:

Criteria/Formulation/Materials of Test Kit

Specific and crucial criteria were met, see table 1. The goal was to provide prepared materials in the test kit which allows onion producers to quickly analyze sweet onions at the onion packing house with same day results. This goal was met by prepared reagents provided in the packaged kit. In each kit, a 20 mL vial of 2, 4 – DNPH in 1.0 N HCl, along with a 20 mL vial of 1.5 N NaOH solution is

included. This eliminates the need for the onion processor to prepare the reagents themselves, which can be both time consuming and potentially hazardous. Another criterion was the stability of the prepared reagents. All reagents used in this procedure are stable in solution, as they will be packaged in the test kit. The 2, 4 – DNPH solution provided in the kit can be stored for extended periods in a cool, dry place with temperatures not exceeding 160˚C (*17*). Similarly, the NaOH reagent provided should be stored in a cool, dry place with temperatures not exceeding 102˚C (*18*). These reagents at the prepared concentrations are not considered hazardous waste (*19*). After the samples are processed (resulting in further dilution), these materials can be disposed of down a sink (*19*). These solutions (as packaged in the test kit) are stable at room temperature. It is important that general handling safety precautions be considered (latex/nitrile gloves, safety eyewear) however do not call for extreme measures.

The goal was to produce a method that can analyze pyruvic acid in the visible range so that a color char could be used if an onion processing facility does not have a spectrophotometer. This goal was met, and notable sensitivity was determined at 515 nm with a percent recovery of 104% ± 10.4% (table 8). Recovery was calculated after duplicate samples were spiked with a known standard prior to reagent addition but after the homogenization and filtration step. Results satisfy the criteria for recovery of 80-120%. Perhaps the most important criterion in the development of the test kit is the ability of the onion processor to analyze sweet onions without the use of analytical instrumentation. This goal was

met, and any sample analyzed using the test kit can be compared to a previously formulated colored color chart (figure 7) included in the kit. A sample can therefore be compared to the color chart provided in the test kit allowing an onion processor to identify which onions are sweet (lower concentrations), and which are more pungent (higher concentrations).

The onion processing facility will need a few additional resources to use in conjunction. The on-site facility would need to utilize their own pipettes (glass or automatic) with the capacity to measure 25 µL and 1 mL sample volumes, test tubes (recommended 16x100 mm borosilicate glass culture tubes), an analytical balance, knife, cheese cloth, other cloth filter, or strainer, a water bath capable of sustaining a temperature of 37 ± 2° C, a blender/homogenizer, and have access to distilled water. The filtration step for the test kit differs from that of the laboratory procedure; however, since the onion processors will not be using spectrophotometry as a means of quantification of pyruvic acid, the turbidity of the onion filtrate does not become a factor in the final color; however it might contribute to variability as discussed previously.

Cost Analysis

Reducing the cost of analyzing sweet onions was the underlying goal of this research. Once the prototype was developed, the cost to produce it was compared to the current cost of analyzing sweet onion as performed by the Texas AgriLife Extension and Research Cooperative. Current costs per sample are $60 (assuming cooperative rates, additional charges for non-coop members do apply but were not included in this analysis) and do not include shipping the

samples to Texas. The shipping rate can vary based on the weight/size of the package, as well as the delivery priority, but can range from $4.90 to $25.15 for a standard 24x12x12 box weighing 20 pounds (*20*). Labor expenses are also considered in the total cost. An onion worker making approximately $15.00 (plus 50% additional costs to the employer) can perform the test within an hour; therefore the labor cost per sample is $22.50. Using the same hourly rate to compare results to a full day of analysis, staff at the Texas AgriLife Research & Extension Cooperative would cost $180.00 to prepare and analyze the same number of samples. A cost analysis for the development of a test kit based on this research was performed (table 9). The total cost to manufacture and produce one test kit is $29.21. One test kit can analyze 20 onions; therefore the cost per onion sample is approximately $1.46. At this rate, 41 samples could be analyzed by an onion production facility using the test kit for the same price as the Texas Co-op currently charges for one single sample. The reduction is cost is significant.

Future Research

Further studies need to be performed. More data representing variation for pyruvic acid concentrations of stored samples for a known period of time as well as variation within a standard protocol are required (*21*). In addition, more robustness studies must be performed to validate the method developed in this research project. Increasing the number of onions per one sample, such as ten onions blended together, would reveal a greater representation of sweetness in a population of onions and should be tested for accuracy. Due to variability from

one onion sample to another, it is recommended to blend several samples at once for a wide representation of the crop being analyzed. A thorough examination of background pyruvic acid is relevant. Considering the data from this research differs from the literature values (by approximately 1 µmol/g), more trials should be performed to reduce the presence of background pyruvic acid. Overall, more replications of this method should be performed for statistical analysis. One potential experiment is to purchase a batch of onions from local grocery stores, and send half the batch to Texas for analysis. Samples should be tested simultaneously using this method and results compared to those from Texas. If the data is determined to be significantly similar, the proof-of-concept will be validated and the test kit can be developed for use within the onion industry.

Conclusions

After reviewing the results and goals of each of the two objectives within this research project, we can conclude a proof-of-concept. Objective 1 sought to determine a colorimetric method to determine enzymatically produced pyruvic acid using a spectrophotometric assay with recoveries of 104%. The laboratory method is reproducible. A SOP is currently awaiting authorization so the Anderson laboratory can continue analysis, future studies, quality assurance, and production of the reagents and other materials provided in the test kit. Objective 2 was the development of the test kit. A prototype is available as a product of this research. A video demonstration is available via the extension outreach program within the Food Safety and Environmental Stewardship Program at Oregon State

University. Finally, any onion processor can use this cost efficient, fast, field test kit to analyze pungency in an effort to increase the quality of their onion crops, or to simply identify which onions meet the criteria of a sweet onion so they may market them as such.

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18. MSDS Sodium Hydroxide. http://www.jtbaker.com/msds/englishhtml/s4034.htm
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Figure 1: *Formation of enzymatically produced pyruvic acid*

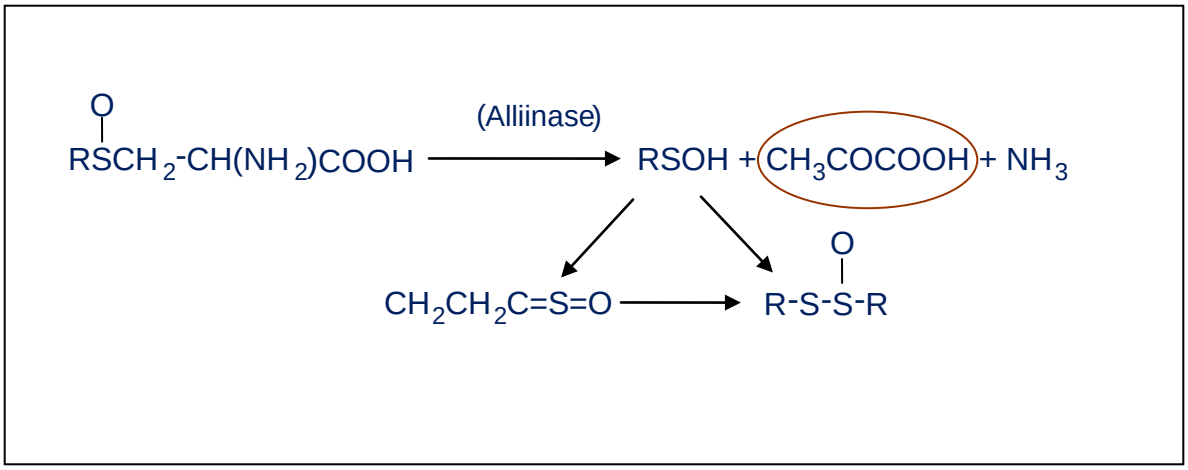

Figure 1: Demonstration of the enzymatic formulation of pyruvic acid from flavor precursors. Pyruvic acid is circled. R = flavor precursors (PrenCSO, PSCO, MCSO)

Figure 2: *Standard/calibration curve at 515 nm*

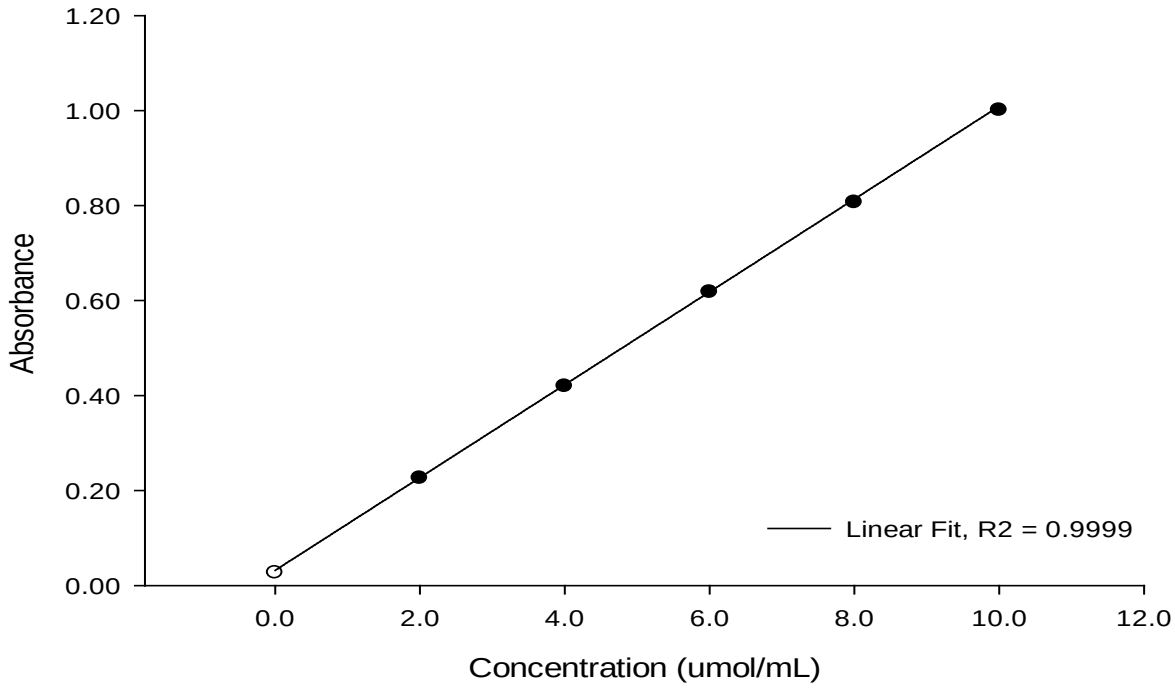

Figure 2: Standard curve using known concentrations of pyruvic acid, sodium salt, 99%+. $R^2 > 0.990$ for all assays

Figure 3: *Standard curve at 420 nm*

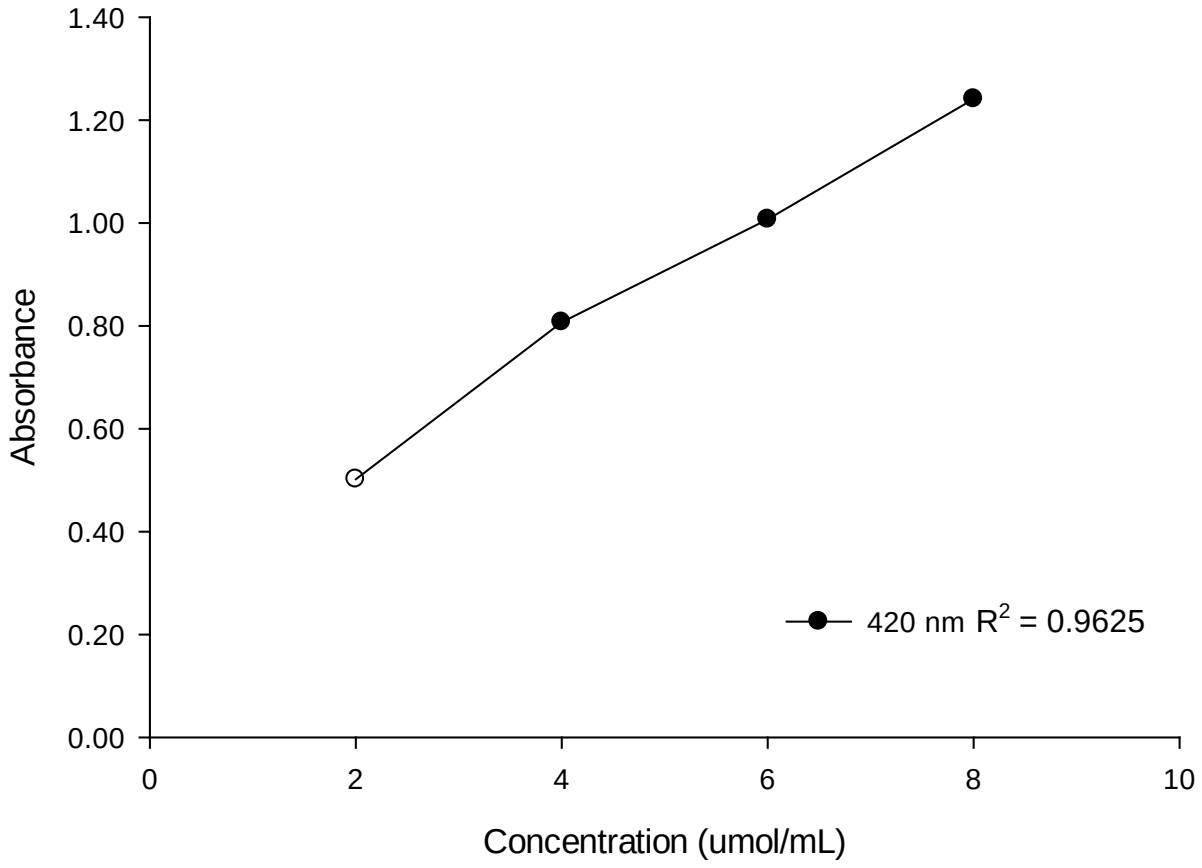

Figure 3: Standard curve at 420 nm. Absorbance (measured at 420 nm) vs. concentration at does not indicate a total linear fit, as indicated by the R^2 value. In addition, absorbance values are greater than 1 may result in deviation from Beer's Law. Absorbance values vary at 420 nm.

Figure 4: *Standard curve at 445 nm*

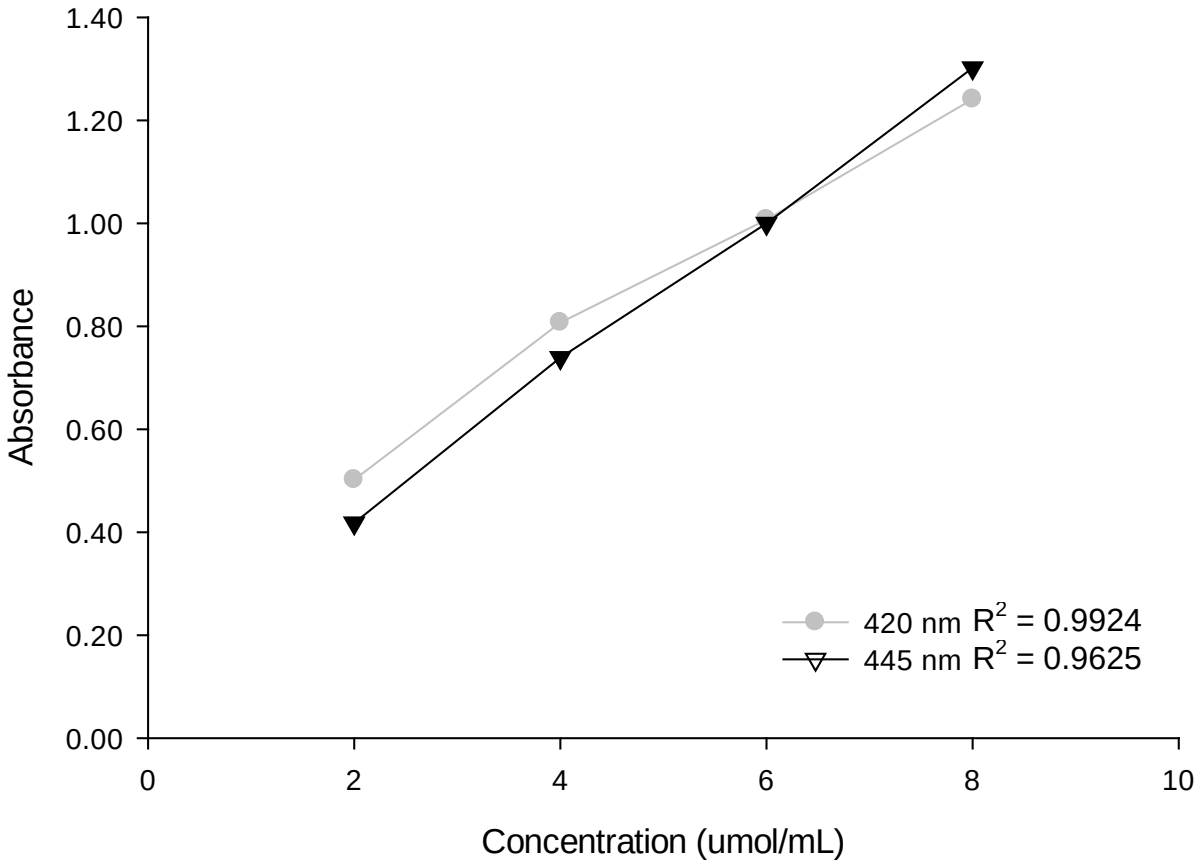

Figure 4: Standard curve at 445 nm. Similar to figure 3, absorbance (measured at 445 nm) vs. concentration at does not indicate a total linear fit, as indicated by the R^2 value. In addition, absorbance values are greater than 1, which does not indicate Beer's Law is applied. Absorbance values vary at 445 nm.

Figure 5: *Onion Sensory vs. Concentration*

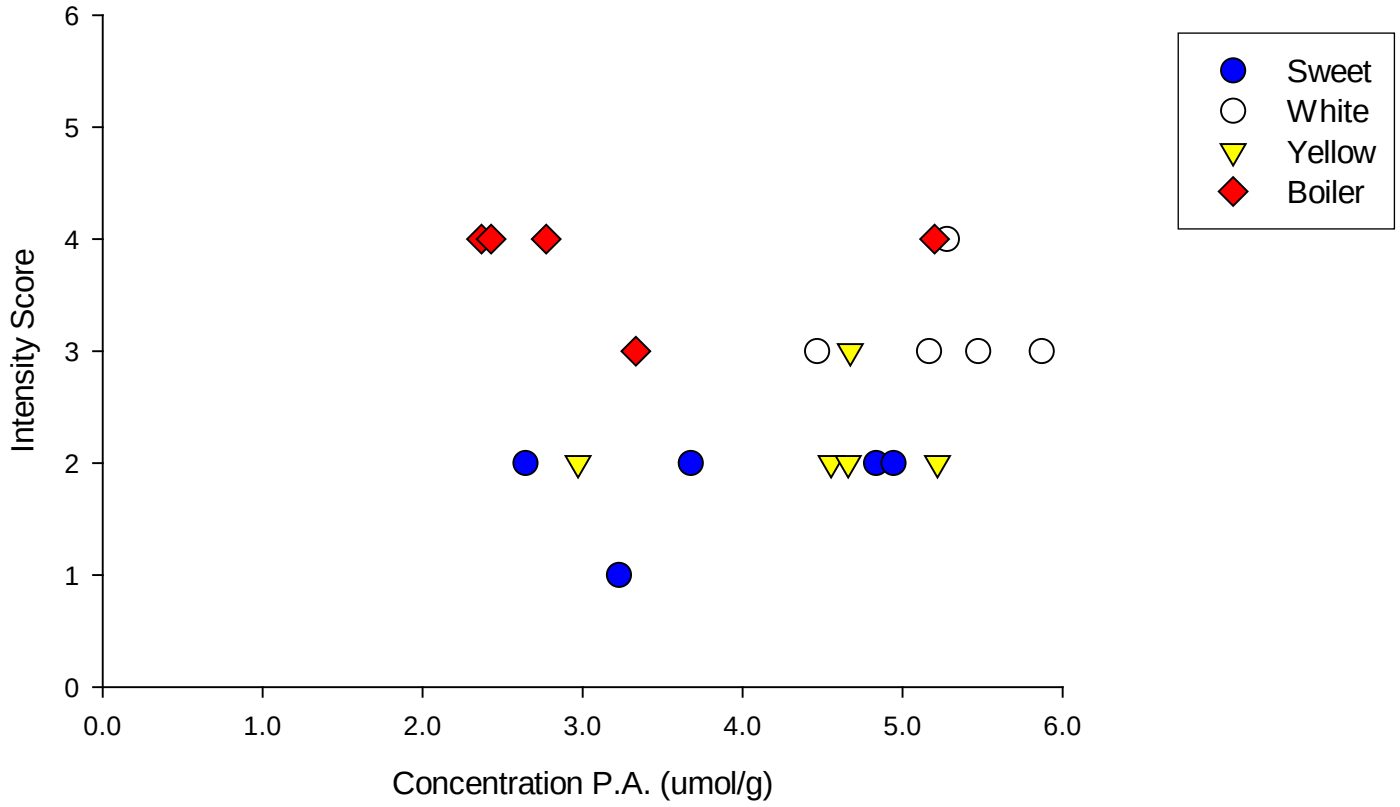

Figure 5: Comparison of sensory score to concentration of pyruvic acid in tested samples. Each value indicates a single sample and its sensory score. Limitations to consider are one panelist responding to aroma stimulus, therefore results are variable.

Figure 6: *Pyruvic acid reaction time*

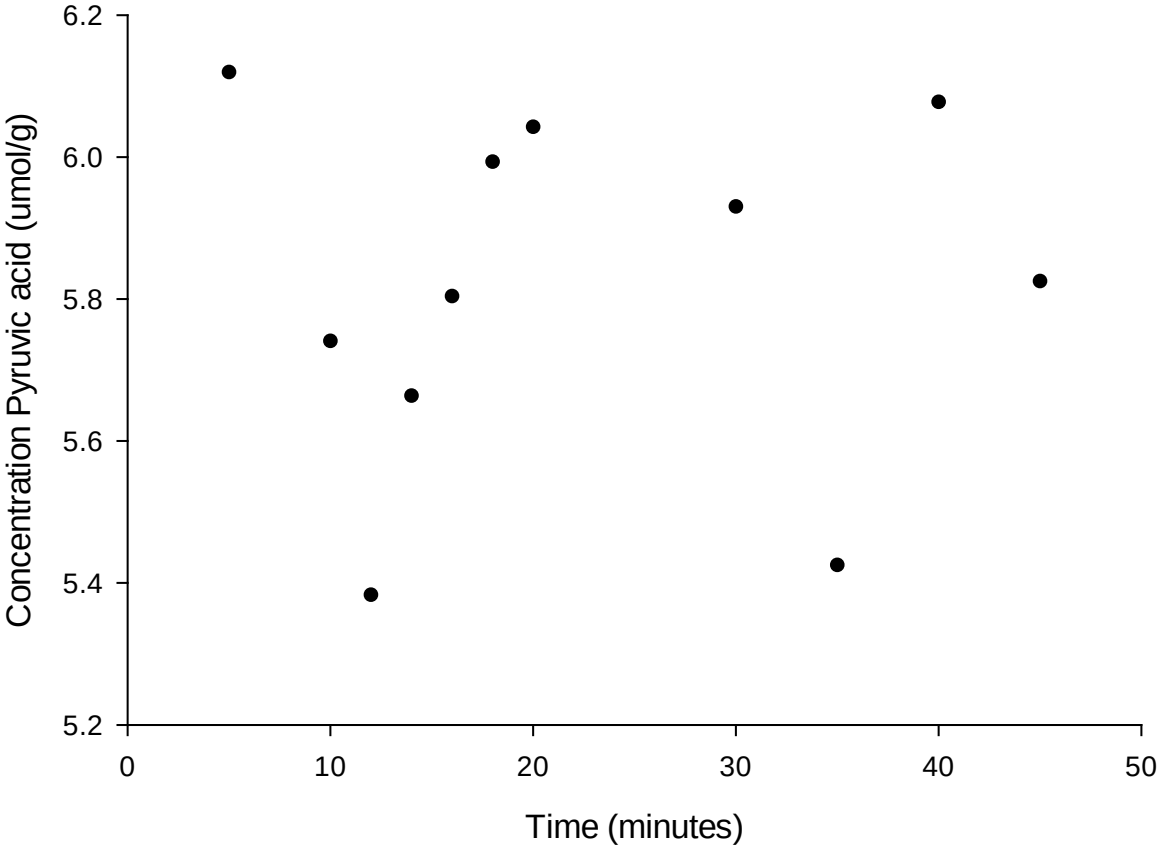

Figure 6: Experiment began as a time study to determine time required for optimum pyruvic acid accumulation in an onion homogenate. Results are not conclusive as filtering did not stop the enzymatic reaction. This figure provides data concerning total pyruvic acid for one yellow onion. Average concentration values are 5.82 ± 0.25 µmol/g. Note: Y-axis range 1 µmol/g.

Figure 7: *Color chart included in the test kit (prototype)*

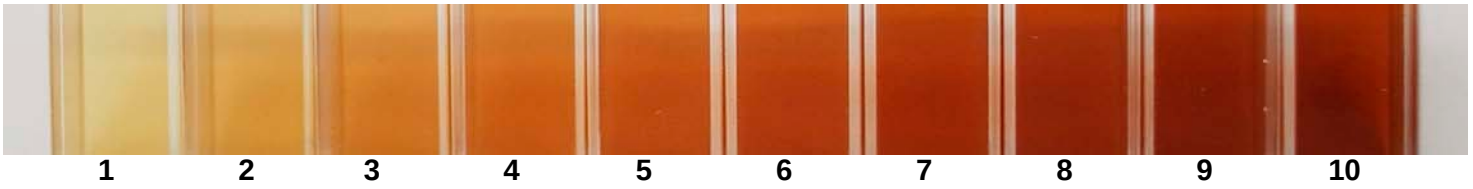

Figure 7: Color chart

Table 1: *Criteria for final product, test kit*

	Ease of Prep	**Stability**	**Non-Hazardous**	**T**	**Visible (nm)**	**% Recovery**	**Final Product**
Ideal Conditions	Quick Prep: 10 minutes or less (daily)	Stable at conditions: T, mp, bp, vp, reactivity	Dispose in general waste container and/or rinsed down the sink	25°C or RT	420-515	80-120	Color of solution determined & compared to color chart
Minimal Acceptable Conditions	2 hour prep time (weekly)	Stable at conditions: T, reactivity	Disposed in hazardous waste containers for later collection	0° - 35°C	400-700	60-120	Lab will need a spectrophotometer

Table 1: Criteria created in the formulation of this research project.
T=temperature, mp=melting point, bp=boiling point, vp=vapor pressure, RT=room temperature, 25˚C, nm=nanometers

Table 2: *Sensory score chart*

Score:	Evaluation:
6	Extremely strong
5	Very strong
4	Strong
3	Distinct
2	Slight
1	Very slight
0	Neutral (no intensity present)

Table 2: Sensory scale of cut onion samples. Each sample was rated based on intensity of aroma and tearing effect.

Table 3: *Standard curve (R^2 values)*

Standard Curve (R^2 values)	
July 23, 2009	0.9997
August 7, 2009	0.9979
August 12, 2009	0.9998
August 13, 2009	1.0000
August 18, 2009	0.9998
August 24, 2009	1.0000
August 25, 2009	0.9998
August 26, 2009	0.9995
August 31, 2009	0.9994
September 11, 2009	0.9999
September 21, 2009	0.9999
September 22, 2009	1.0000
October 7, 2009	0.9995
October 10, 2009	0.9999
October 12, 2009	0.9997
October 21, 2009	0.9992
October 22, 2009	0.9999
October 24, 2009	0.9998
Average	**0.9997**
S.D	**0.0005**

Table 3: Raw data from standard curve assays throughout experimental design process. Results show an R^2 value of 0.990 ran for each assay.

Table 4: *Results from onion assay (n = 5)*

Varietal	Average TPA (µmol/g)	Average BPA (µmol/g)	Average EPA (µmol/g)
Sweet	5.90 ± 0.24	1.88 ± 0.43	4.03 ± 0.49
White	6.59 ± 0.39	1.77 ± 0.35	4.83 ± 0.35
Yellow	7.44 ± 0.31	1.58 ± 0.38	5.86 ± 0.08
Boiler	6.46 ± 0.32	1.58 ± 0.29	4.88 ± 0.39

Table 4: Results from one analysis of 5 onions of each varietal. Values reported are averages and standard deviation of 5 onions. Sweet onions analyzed in this research do classify as sweet onions according to the industry standards where they define sweetness by onions having 1 – 4 µmol/g pyruvic acid.

Table 5: *Results from onion assay (n = 15)*

Varietal	Average Pyruvic acid (umol/g)
Sweet	4.05 ± 0.48
White	4.85 ± 0.09
Yellow	5.85 ± 0.06
Boiler	5.02 ± 0.09

Table 5: Results from a triplicate analysis of 5 onions from each varietal. Values reported are averages and standard deviation of 15 onions.

Table 6: *Results – total pyruvic acid values compared to literature*

Varietal	Schwimmer & Weston (1961)[1] (µmol/g)	Anthon & Barrett (2003)[1] (µmol/g)	Merrill, et. al. (2009)* (µmol/g)
Sweet (Vidalia)	4.47 ± 0.24	3.61 ± 0.24	-
Sweet (Melody)	4.30 ± 0.15	3.43 ± 0.14	-
Sweet**	-	-	5.90 ± 0.24
White	6.54 ± 0.65	6.98 ± 0.69	6.59 ± 0.39
Yellow	12.34 ± 1.17	10.81 ± 1.11	7.44 ± 0.31
Boiler	-	-	6.46 ± 0.32

[1] Anthon & Barrett (2003) J Sci Food Agric **83**:1213

* Results not published
** Unknown origin

Table 6: Results compared to literature values reported by Anthon & Barrett (2003). Data provided in this study were provided by Anthon & Barrett who used the same samples for the two methods. n values are unknown for the literature data are not known. Results from this research do compare to literature values by following the same trends where sweet onions have the lower concentration of pyruvic acid.

Table 7: *Results – background pyruvic values compared to literature*

Varietal	Schwimmer & Weston (1961)[1] (µmol/g)	Anthon & Barrett (2003)[1] (µmol/g)	Merrill, et. al. (2009)* (µmol/g)
Sweet (Vidalia)	0.76 ± 0.03	0.19 ± 0.01	-
Sweet (Melody)	0.78 ± 0.06	0.16 ± 0.02	-
Sweet**	-	-	1.88 ± 0.43
White	0.26 ± 0.02	0.21 ± 0.01	1.77 ± 0.35
Yellow	1.27 ± 0.27	0.32 ± 0.13	1.58 ± 0.38
Boiler	-	-	1.58 ± 0.29

[1] Anthon & Barrett (2003) J Sci Food Agric **83**:1213

* Results not published
** Unknown origin

Table 7: Results compared to literature values reported by Anthon & Barrett (2003). Data provided in this study were provided by Anthon & Barrett who used the same samples for the two methods. n values are unknown for the literature data are not known. Data from background analysis are higher based on this research, and warrant further investigation.

Table 8: *Results – percent recovery*, *over-spike*

Sample	Total Pyruvic Acid (µmol/g)	Background Pyruvic Acid (µmol/g)	Enzymatic Pyruvic Acid (µmol/g)	% Recovery
FMBLR01	7.366	1.861	5.505	-
FMBLR01 – MS	18.326	12.881	5.445	99
FMBLR02	6.462	1.392	5.108	-
FMBLR02 – MS	18.482	12.572	5.949	116
FMBLR03	7.204	1.634	5.570	-
FMBLR03 – MS	18.117	12.732	5.385	97

Average (3 replicates)	104%
S.D. (3 replicates)	10.4%

Table 8: Samples were spiked (MS) with a known standard (6 µmol/g) and analyzed for recovery. Spiked samples were divided by unspiked samples and multiplied by 100 for total percent recovery. An average and standard deviation produced a result of 104% ± 10.4% recovery.

Table 9: *Results – percent recovery, check standards*

Sample	Standard Sodium Pyruvate (µmol/g)	Average Recovered Standard* (µmol/g)	% Recovery
1	2	1.98	99.0
2	4	3.89	97.3
3	6	5.91	98.5
4	8	7.86	98.3
5	10	9.72	97.2

* n = 3

Average	98.0%
S.D.	0.78%

Table 9: Standards were run as check standards and analyzed for recovery. Recovered standard samples were divided by the known standards and multiplied by 100 for total percent recovery. An average and standard deviation produced a result of 98.0% ± 0.78% recovery.

Table 10: *Cost analysis for final product, test kit*

Material	Total Cost	Considerations	Cost per item	Quantity in Test Kit	Cost per test kit
Reagent: NaOH	$35.70 (500g)	1.2g needed for 20mL solution	$0.09	1	$0.09
Reagent: DNPH	$29.00 (25g)	0.005g needed for 20mL solution	<$0.01	1	$0.01
Reagent solution: HCl	$52.90 (500mL)	0.6mL needed for 20mL solution	$0.06	n/a	$0.06
20mL Amber Vials	$170.40 (144 vials)	2 vials needed per kit	$1.18	2	$2.37
Disposable Pipettes	$7.99 (100 in pack)	2 p/ sample for DNPH & NaOH, 20 samples p/20mL solution	$0.08	40	$3.20
Color Chart					
Paper	$6.29 (500 sheets)	2 color charts per 1 sheet of 8.5 x 11 paper	<$0.01	1	$0.01
Lamination cost	$3.00 (p/sq.ft)	2 color charts per 1 sheet of 8.5 x11 paper	$0.97	1	$0.97
Labor	$22.50 p/hour	20 hours to analyze 20 solutions in test kit	$22.50	n/a	$22.50
Total Cost:					**$29.21**
Samples per kit:					**20**
Cost per sample:					**$1.46**

Table 10: Cost analysis to produce the final product, a onion sweetness test kit. Current industry charges $60/sample for onion analysis, and require a cooperative membership to receive that price. This test kit reduces the total cost of one sample to $1.46.

Appendix A: *Standard Operating Procedure (SOP)*

Title: Determination of Pyruvic Acid in Onion Tissue

Scope

This method describes analysis of pyruvic acid in onion tissue. Pyruvic acid is measured by spectrophotometry with known concentrations of standard. The instrumental analysis is completed using a UV/Vis Spectrophotometer. Detection limits are determined routinely, but typically are 0-20 μmol.

The samples should be maintained at 4°C at the time of collection and stored frozen.

Status

This document is considered current standard operating procedure of the Food Safety and Environmental Stewardship Laboratory when management approval is documented by signature below. This Standard Operating Procedure is effective on the date of approval signature and supersedes all previous versions.

Filepath:

Approved	_____	**Director**	_____
	Kim A. Anderson, Ph.D.	Title	Date
Historical File	_____		_____
	Signature/Initials		Date

I. Equipment and Apparatus

- **A.** Blender
- **B.** Styrofoam cups
- **C.** Cheese Cloth
- **D.** Mettler Balance, Model AG245 and Model PG5002-S (or equivalent)
- **E.** UV/Vis Spectrophotometer (or equivalent)
- **F.** Water bath
- **G.** Cutting board, knife, weigh boats or paper, Ziploc bags
- **H.** Automatic pipettes (100uL, 1000mL)
- **I.** Test tubes (recommended: 16x100 mm borosilicate glass culture tubes)

Note: Organic strip and bake all glassware, containers and vials before use (See SOP 2110 – Laboratory Container Cleaning procedure).

II. Reagents

- **A.** 18 MΩ•cm water, EASYpure UV, Barnstead.
- **B.** 0.25 g/L DNPH in 1 N HCl
- **C.** 1.5 N NaOH

III. Standards

- **A.** Primary Solid Standards
  - **1.** 99+% Pyruvic acid, Sodium Salt (or equivalent)
- **B.** Standard Solutions
  - **1.** Analytical stock solutions of the primary standards are weighed and dissolved in water (18 MΩ•cm).
  - **2.** Standard dilutions for fortification shall be prepared at levels that are comparable to estimated sample levels.

IV. Instrument Operating Parameters

V. Sample Preparation

- **A.** Sample Processing
  - **1.** Samples are to be stored at ≤ 4 °C upon receipt.
  - **2.** To prepare for analysis, remove sample from the refrigerator.
    - **a)** Cut the onion longitudinally and cut off both ends
    - **b)** Cut the onion in half and label each half A and B, respectively
    - **c)** Weigh 50g of onion half A and record to the nearest 0.01g.
    - **d)** Weigh 50g of onion half B and record to the nearest 0.01g.
    - **e)** Heat onion half B in the microwave for 1 minute (Power equivalent to 1450 W).
    - **f)** Set aside onion half B for 10 minutes to cool

g) Blend onion half A with equal mL of water to gram of onion fresh weight (ex. A 50g sample should be blended with 50mL water)

h) Let onion slurry A stand for 10 minutes

i) Blend onion half B with equal mL of water to gram of onion fresh weight (as done for onion half A)

B. Extraction

1. Filter onion juice from each sample through 2 layers of cheesecloth into separate and labeled Styrofoam cups. Note: Use new cheesecloth for each sample.

2. Pipette 25 µL clarified onion juice from each sample into separate test tubes

3. Add 1mL 18 MΩ•cm water to each sample

4. Add 1mL 0.25 g/L DNPH solution to each sample

5. Place sample solutions in 37°C water bath for 10 minutes

6. Add 1mL 1.5 N NaOH to each sample

7. Measure absorbance using spectrophotometer, wavelength 515nm

VI. Sample Analysis

A. Calibration standards are used to calibrate the instrument and construct a standard curve.

B. The sample is analyzed for pyruvic acid using the UV/Vis instrumentation and operating conditions listed in the "Instrument Operating Parameters" section.

C. Identify the analyte in the sample by comparing the absorbance of each sample for each wavelength and the absorbance of each standard solution.

D. Calculate concentration using the calculations listed below.

VII. Calculations

A. Fortification levels:

1. *[std. vol. (mL) X std. conc. (µmol/mL] / wt. of sample (g) = fortification level (µmol/g*).

B. Unknown sample levels:

1. External standards are used to quantify the amount of pyruvic acid in the sample.

2. A standard curve is run at the beginning and end of the sample set. All standards are used to determine a linear equation when absorbance vs. concentration is plotted. This equation is used to calculate the amount of pyruvic acid in the extracted sample *(µmol/mL)*. Multiply the instrument concentration by the extract final volume (3.025 mL) to determine the amount of pyruvic acid in the sample (*µ*mol/g).

VIII. Quality Control

A. Each batch run should consist of field samples and appropriate numbers of field or laboratory QC samples (i.e., Matrix Blanks and Fortified Matrix Blanks) to insure batch extraction quality. For a sample set the average QC recovery must be between 70 and 130%. The QC summary consists of the results of all QC samples analyzed including calibration curves with R squared values.

IX. Documentation Requirements

A. A completed bench/QC summary sheet is required for all analysis.

1. Path:

X. Safety and Health

A. Please consult Material Safety Data Sheet (MSDS) information on chemicals and reagents. Personnel performing this method will observe all appropriate Oregon State University laboratory safety procedures.

XI. References

A. Anthon, G.E.; Barrett, D.M.; Modified method for the determination of pyruvic acid with dinitrophenylhydrazine in the assessment of onion pungency. *J Sci Food Agric* 83: 1210-1213 (2003)

XII. Validation

A. This method has been reviewed and validated for pyruvic acid in onion tissue.

B. The validation data is stored in the fireproof cabinet, ALS 1112.

Appendix B: *Instruction manual for test kit*

Instruction Manual for Pyruvic Acid Test Kit

Materials included in this test kit include:

- 1 - 20 mL vial containing color reagent, 2, 4-DNPH in 1 N HCl solution
- 1 - 20 mL vial containing alkaline solution, 1.5 N NaOH
- 1 - color chart with reference concentrations
- 1 - instruction manual and access to instructional video

Materials needed:

- Knife/Cutting board
- Test tubes (Recommended: 16x100 mm borosilicate glass culture tubes)
- Analytical balance capable of measuring weight to the nearest 0.01 mg
- Cheese cloth
- Water bath capable of sustaining 37 ˚C temperature
- Blender/Homogenizer
- Distilled water
- Automatic pipette (capable of measuring100 µL and 1 mL)

Instructions:

1. Cut the ends of the onion and remove any paper-like outer scales
2. Cut the onion into four quarters, collecting one quarter for analysis
3. Weigh one quarter (now the sample) to the nearest 0.01 mg
4. In blender, homogenize sample with equal volume distilled water (ex. 20 g onion with 20 mL distilled water).
5. Let sample homogenate sit for 20 minutes.
6. Filter sample through cheese cloth.
7. Pipette 25 µL onion juice into sample test tube
8. Add 1 mL distilled water
9. Add 1 mL 2, 4-DNPH solution
10. Place in water bath at 37 ˚C for 10 minutes
11. Add 1 mL 1.5 N NaOH solution
12. Compare color produced to chart provided

Color & Concentration:

1 – 4 µmol/g	→	Very mild pungency (sweet)
5 – 7 µmol/g	→	Mild pungency
8 – 10 µmol/g	→	Intermediate pungency
> 15 µmol/g	→	Strong pungency (sharp)